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Do oxalic acid exudates from mycorrhizal fungi influence the uptake of phosphorus by primary producers in the karst critical zone of south west China?

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ABSTRACT

Karst landscapes cover 12% of Earth's continental land area and are often characterised by their nutrient-poor soils. The karst critical zone of south west China, home to 35 million people, is being intensively farmed, despite its highly-degraded and nutrient-limited soils. Phosphorus, a key nutrient for plant growth, is thought to be a limiting nutrient in karst soils as a result of negligible concentrations found in limestone bedrock and the influence of anthropogenic farming practices. This research sought to understand if organic acid exudates from mycorrhizal fungi, found in >80% of plants, altered the uptake of phosphorus by primary producers in karst soils. To answer this, a sequence of plant-based experiments were conducted using soils collected from Chenqi subcatchment, Guizhou Province. Oxalic acid treatments were added to soils to simulate the action of organic acids exuded by mycorrhizal fungi, to identify if non-labile phosphorus was being broken down into labile species. SEM EDS analysis of soil from Chenqi was used to identify the phosphorus species present in the soil, to better our understanding of the phosphorus cycle in karst environments. The results indicate that soils in Chenqi subcatchment are severely limited with respect to phosphorus, and that this impacts detrimentally upon the overall health and growth of plants. Statistical analysis suggests that oxalic acid does not significantly increase the concentration of phosphorus taken up by plants in karst soils. This research is part of the NERC-NSFC-Newton funded SPECTRA project, investigating soil processes and ecological services in the karst critical zone of south west China, an international project involving research institutions in the UK and China. The results of this research will be collated with other SPECTRA project findings, to be used in improving understanding and management of the response, resilience and recovery of the south west China karst.

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Finally, I would like to dedicate this to Liz, who inspired my love of Geography, and who sadly never got to read this. You are sorely missed by so many.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicates by a specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: V.Hussey DATE: 07/09/2018

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1.0. INTRODUCTION

Almost 35 million people in south west China rely upon karst environments as a source of food and potable water, and with these fragile landscapes subject to rapid degradation and desertification, there is significant uncertainty over the future of sustainable farming in rural karst areas. Karst environments are known to cover 12% of the Earth's continental area (Hartmann et al., 2014), and are often characterised by nutrient-poor soils of varying depths, as a result of the underlying carbonate bedrock that is highly susceptible to erosion and chemical weathering processes (Bull, 2005).

Human activity has shaped many karst environments across the world, with increased rates of deforestation required to meet commercial farming needs resulting in soil erosion and degradation, which can take hundreds of years to recover and revegetate. As a direct consequence of soil erosion, many karst regions have nutrient-poor soils which limit primary production. Traditionally, mineral fertilisers comprising of nitrogen, phosphorus and potassium (known as NPK fertilisers) have been applied to improve total crop yield, however this also results in eutrophication of freshwater systems (Simmons, 2000). Eutrophication, the process of the pollution of waters by elevated concentrations of nutrients such as nitrogen and phosphorus, alters the primary productivity of an ecosystem and can cause impacts such as deoxygenation of waters (Simmons, 2000). A balance must be struck between ensuring adequate nutrient concentrations in karst soils and limiting the impact of eutrophication of freshwater ecosystems. To achieve this, more sustainable methods of increasing nutrient availability in soils is required, in place of the traditional excess application of NPK fertilisers which is used to alleviate issues associated with nutrient-limited soils as are characteristic of karst environments.

Phosphorus is of particular concern in karst regions, given its role as a primary limiting nutrient in marine, freshwater and terrestrial environments (Elser et al., 2007). In many cases, phosphorus is present in soils, but is held in a non-bioavailable form that cannot be used by plants for key processes such as growth an reproduction (Holford, 1997; Schachtman et al., 1998). The low concentrations of bioavailable phosphorus in soils augments the aforementioned use of NPK fertilisers to boost soil concentrations. Karst environments, such as the south west China karst, are often subject to elevated rates of soil erosion which in turn removes phosphorus from the terrestrial environment. Erosion and runoff of sediments, primarily consisting of topsoil and phosphorus-rich fertilisers, are not only wasteful and costly but also drives eutrophication of freshwater (and subsequently marine) ecosystems. In addition to the considerations regarding eutrophication, the excess application of phosphorus in fertilisers is problematic due to the finite and complex nature of phosphorus as a natural resource (Childers et al., 2011). Phosphorus reserves are being rapidly depleted, due to the increases in agricultural production required to meet the demands of global population growth; in the USA, one of the largest exporters of

phosphorus, it is thought that domestic phosphorus reserves will be depleted in the next 15 to 25 years (Stewart et al., 2005). Given this rapid depletion of global phosphorus reserves, there is a need for increased research and understanding of how phosphorus in soils can be accessed and used more efficiently, reducing the need for superfluous application of fertilisers.

One method by which phosphorus concentrations in soils can be increased, is through the action of mycorrhizal fungi. These fungi inhabit the root systems of over 80% of all plants (Smith and Read, 2008; Wang et al., 2010), and release chelating compounds such as oxalic, lactic and malic acids. These compounds have been shown to break down non-labile and non-bioavailable phosphorus, such as apatite, into soluble and available inorganic species which can subsequently be used by primary producers (Jones, 1998; Yadav and Tarafdar, 2003). There is limited understanding of some of the interactions between mycorrhizal fungi, organic acids and phosphorus, but there is evidence to suggest that the addition of oxalic acid to soil or rock can increase the concentration of bioavailable phosphorus suitable for uptake by plants and other primary producers (Kpomblekou-A and Tabatabai, 2003; Panhwar et al., 2013).

The SPECTRA project is a NERC-Newton Fund international research project, which brings together scientists from the UK and China. SPECTRA has a key focus upon soil processes and ecological services in the karst critical zone of zone of south-west China, with a key aim of better understanding the resilience, recovery and response of the south China karst to anthropogenic perturbation and activity. The four workpackages within SPECTRA are interlinked, and cover the following ideas:

- Soil erosion and redistribution
- Distribution of biota, including plants, fungi and microbes
- Rock weathering, and subsequent elemental release and processes surrounding soil formation
- Soil nutrient pools and fluxes, including phosphorus, nitrogen and organic carbon

These findings from these workpackages will combine to generate a wide range of data on the south China karst, including biogeochemical cycles, ecosystem structure and interactions and geological formations and processes. It is crucial that biogeochemical cycling of carbon, nitrogen and phosphorus in karst ecosystems is better understood if these environments are to be managed in a sustainable manner. Soil degradation and nutrient exhaustion must be controlled if there is to remain a future in agricultural production in such highly-degraded environments. It is hoped that this improved understanding of such a complicated environment can be used to inform policy-making and management of the south China karst ecosystem, to both restore and protect the environment in addition to optimising agricultural yields and ensuring sustainable livelihoods for the 35 million inhabitants.

As part of the SPECTRA project, this research aims to fill some gaps in the current literature and fill the knowledge base surrounding phosphorus in karst environments. Interactions between organic acids and phosphorus in karst regions have been poorly explored, and there is little understanding of the role of organic acids in mobilising phosphorus in otherwise nutrient-limited regions. This research seeks to understand if oxalic acid, known to be exuded from mycorrhizal fungi, can increase the uptake of phosphorus by plants in soils sampled from the karst critical zone of south west China. To answer this question, a suite of plant-based experiments was designed to determine the impact of oxalic acid on phosphorus uptake by *Erigeron acris* seedlings, using soils sampled from the Chenqi subcatchment, located in Guizhou Province. Furthermore, SEM EDS imaging of soil samples was employed to identify the presence of phosphorus in soils from Chenqi, and to establish the species of phosphorus most commonly found in karst environment soils. Given the primary land-use of karst regions such as Chenqi as areas of subsistence and commercial agriculture, analysis of the overall plant health and growth quality were also assessed as such a factor is important in maintaining livelihoods for many people living in the south west China karst.

2.0. CURRENT LITERATURE

As is outlined in Section 1.0., there are several motivations for conducting research into phosphorus limitation in karst critical zones that are heavily relied upon for agricultural cultivation. To better understand these motivations and to provide an insight into the current literature, the following sections will explore a range of topics, including: the phosphorus cycle; the role of mycorrhizal fungi in plant-nutrient interactions, and karst regions and the complex pressures associated with them.

2.1. PHOSPHORUS IN THE NATURAL ENVIRONMENT

2.1.1. PHOSPHORUS: STRUCTURE, FORM AND ABUNDANCE

Phosphorus, a key nutrient for all living organisms, is a naturally-occurring mineral found in the earth's crust, with the current abundance measured at 0.12% (Hanrahan et al., 2004). It is essential for growth and maintenance of living cells (Elser et al., 2007; Smits et al., 2012), in particular in the formation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), structuring phospholipids and cell membranes, and the formation of metabolic agents such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), the most common biomolecule in the natural world (Magid et al., 1996; Marschner, 1995; Schlesigner, 1991; Stewart and Tiessen, 1987).

Phosphorus is held in a variety of forms within the natural environment: organic, soluble inorganic, insoluble inorganic and surface adsorbed (Dalai, 1977; Larsen, 1967). Phosphorus is cycled between rock, soil and water, with its form and abundance dependent on soil type, plant species and other environmental and anthropogenic influences (Anderson and Magdoff, 2005; Bieleski, 1973; Chien and Menon, 1995). Most phosphorus in the natural environment is held in rocks and soils, with low concentrations found in water; mineral forms of phosphorus, such as apatite, have a very low solubility in water, and therefore are more commonly associated with the terrestrial landscape (Hanrahan et al., 2004). In relation to uptake by primary producers, phosphorus can be found in either bioavailable or non-bioavailable species; bioavailable phosphorus refers to phosphorus held in a labile form, which can be immediately accessed and used by primary producers. Conversely, non-bioavailable species of phosphorus are those found in the non-labile form, and so must undergo transformative biochemical processes to become bioavailable, labile species, that subsequently can be used by primary producers. With most phosphorus in the natural environment held in non-bioavailable particulate form, it is commonplace that phosphorus is the main nutrient that limits primary production (Schindler, 1977; Smith et al., 1986).

In natural environments, the weathering of rock is the dominant source of phosphorus for living organisms (Smits et al., 2012). Phosphorus is present as apatite, vivianite, wavellite and phosphorites (Budavari, 1989; Hanrahan et al., 2004); the most important of these weathered minerals is apatite (Guidry and Mackenzie, 2000; Peltzer et al., 2010; Smits et al., 2012; Walker and Syers, 1976), as it accounts for >95% of all phosphorus found in the earth's crust (Hallberg, 1992; Jahnke, 1992; Schlesigner, 1991; Stevenson, 1986). The apatite reservoir refers to multiple compounds, including: fluoroapatite, hydroxyapatite, carbonate-hydroxyapatite and francolite (Van Straaten, 2002). Different forms of apatite are characterised by the other elements present within the structure; those with a higher carbonate content, such as francolite, will solubilise more easily than others, thus releasing more plant-available phosphorus into the soil (Anderson et al., 1985). Igneous rocks are rich in fluoroapatite, whilst authigenic carbonate-fluoroapatite is the main phosphorus-bearing mineral in sedimentary rocks (Filippelli, 2008).

Table 1 - The uses of mined phosphorus, and the associated proportion of the global total used for each purpose. Data sourced from Prud'homme (2010) and Schröder et al. (2010).

Phosphorus Use	Proportion of Mined Phosphorus (%)
Mineral Fertiliser	82
Animal Feed	7
Detergents	5
Speciality Applications (e.g. lighting; food-grade phosphorus; lighting; metal treatment)	4
Food Additives	1-2

Phosphorus deposits are located across the world and are mined to serve a range of agricultural, domestic and industrial purposes, as are displayed in Table 1. There are deposits and reserves of phosphorus located in all continents excluding Antarctica, but these are rapidly depleting. Stewart et al (2005) state that, by using average phosphorus mine production data from 1997-2001, they estimate that world deposits will last until 2095, and world reserves will last until the year 2345. Such predictions work on the basis of fixed socio-economic factors, and therefore given the projections in population growth over the 21st Century, these estimations are likely to overpredict the longevity of the resources. As a limiting nutrient in most soils, phosphorus is usually mined and subsequently added to soils as mineral fertilisers; given its finite nature, a more sustainable attitude to phosphorus within soils and agriculture must be adopted before resources are too severely depleted.

Previous research indicates that the total phosphorus concentration found in soils ranges from 100 to 3000mg kg^{-1} (Condron et al., 2005), but only 1 to 5% of this is thought to be in a soluble, bioavailable form (Hayman, 1975; Molla et al., 1984; Pierzynski et al., 2005). Therefore, interactions between recalcitrant fractions and the surrounding environment occur in order to release bioavailable phosphorus into the soil (Condron et al., 2005; George et al., 2002; Tang et al., 2006; Tarafdar and Jungk, 1987). Often, this involves the action of soil microorganisms, which alter the soil environment to promote the solubilisation of inorganic phosphorus (Taha et al., 1969); some higher plants have also evolved to better absorb phosphorus from nutrient-limited soils (Ozanne, 1980), which are further explained in Section 2.2. The concentration of phosphorus in the soil solution phase in most soils is frequently less than that of many micronutrients such as iron, manganese and copper (Epstein, 1972; Fried and Broeshart, 1967). The soil macronutrient ions, excluding phosphorus, are usually found in soils in concentrations of 10^{-3}M to 10^{-4}M, whilst phosphates are often found to have the lowest concentrations, measuring 10^{-6}M (Goldstein, 1986). It is therefore unsurprising that phosphorus is the main limiting nutrient for plants, and it is this deficiency in soil solution phosphorus that remains predominantly responsible for preventing the maximum agricultural yield from being obtained (Goldstein, 1986).

2.1.2. ORGANIC AND INORGANIC PHOSPHORUS

Phosphorus is held in a diverse range of forms within the natural environment, encompassing both organic and inorganic species (Dalai, 1977; Larsen, 1967). The respective abundances and concentrations of these species is related to characteristics of the terrestrial or freshwater environment, such as pH, soil phosphorus concentration and soil type (Anderson and Magdoff, 2005; Bieleski, 1973; Chien and Menon, 1995). In most soils, phosphorus originates from the weathering of apatite minerals, where phosphorus is bound to calcium, iron, chlorine or a hydroxide group (Pierzynski et al., 2005). Weathering of soils increases the abundance of aluminium- and iron-bound phosphorus species, in addition to higher concentrations of organic phosphorus (Pierzynski et al., 2005).

Organic phosphorus refers to any phosphorus species containing a covalent bond to carbon; these organic compounds are classified based on the nature of the phosphorus bond within their structure (Condron et al., 2005). Between 30 and 90% of phosphorus found in soils is held in the organic form (Harrison, 1987; Oberson et al., 1996; Stevenson, 1986), which itself is not bioavailable. Most forms of phosphorus are not immediately bioavailable, and so can only be used by primary producers having undergone mineralisation or another biochemical process (Stewart and Tiessen, 1987). It is these processes that create the phosphorus cycle and see the transition of phosphorus between organic and inorganic phases.

The organic phosphorus fraction within soils is made up of a range of compounds: orthophosphate esters, phosphonates and anhydrides are all prevalent, depending on the nature of the phosphorus bond (Condron et al., 2005). In most soils, orthophosphate esters, in particular monoesters, are the dominant form of organic phosphorus; they often account for 100% of the total organic phosphorus (Condron et al., 2005). Orthophosphate diesters are another commonly-found form of organic phosphorus, referring to nucleic acids and phospholipids that originate from decayed plant material (Cade-Menun et al., 2000; Condron et al., 2005). Although typically comprising less than 10% of the total organic phosphorus within cultivated agricultural soils, they can constitute more than 50% of the organic phosphorus pool within some forest soils (Cade-Menun et al., 2000). Nucleic acids and phospholipids are some of the few species of organic phosphorus that are immediately bioavailable, and can be used by primary producers without mineralisation occurring (McKercher and Tollefson, 1978).

A further species within the organic phosphorus fraction, microbial biomass comprises between 3 and 24% (Brookes et al., 1984), and includes sugar phosphates, phosphoproteins and mononucleotides (Stevenson and Cole, 1999). Although microbial biomass phosphorus represents only a minor fraction of the organic component, it remains a key actor in phosphorus recycling (Kwabiah et al., 2003). Microbial biomass uses biochemical processes to convert phosphorus from organic to inorganic

species, which are found to be bioavailable and thus available for uptake by primary producers (Stewart and Tiessen, 1987).

Inorganic phosphorus is the most commonly bioavailable form of phosphorus found within most soil systems, and is therefore of vital importance when considering primary production and agriculture (Pierzynski et al., 2005). Orthophosphate (PO_4^{3-}) is the main inorganic phosphorus fraction found in soils solution, of which the main ionic forms are HPO_4^{2-} and $H_2PO_4^-$. These ions are considered to be the primary source of phosphorus for plants and microorganisms, given their bioavailable state (Condron et al., 2005; Pérez Corona et al., 1996). The orthophosphate ions that are taken up by primary producers for use in growth and reproduction must be continually replenished to maintain soil health; this involves the desorption and dissolution of inorganic phosphorus and the mineralisation of organic phosphorus (Condron et al., 2005). Due to their nature as a soluble and labile species, orthophosphate ions are relatively reactive within soils, and therefore often involved in other biochemical cycling processes which reduces the soils bioavailable phosphorus concentration. Through balancing these biochemical processes, a steady concentration of bioavailable phosphorus in soil solution can be maintained.

There are three main groups that soil inorganic phosphorus is categorised into: primary phosphorus minerals, secondary phosphorus minerals and sorbed phosphorus (G.M. et al., 2000; Stevenson, 1986). Primary phosphorus minerals refers to the apatite groups, outlined in Section 2.1.1., whilst secondary minerals are phosphorus species that are bound to calcium, iron or aluminium ions through precipitation (Pierzynski et al., 2005). Secondary phosphorus minerals are formed when bioavailable inorganic phosphorus species react with metal ions in soil solution. The ions present in soil solution vary depending on soil pH: iron, aluminium and manganese are present in acid soils, whilst calcium is more prevalent in alkaline soils, although other regional factors can affect the soil elemental composition. The final form of phosphorus found within terrestrial environments is sorbed phosphorus, present as clays and aluminium or iron oxide compounds (G.M. et al., 2000). These species are insoluble, and so slowly released into the soil solution (Mengel and Kirkby, 1987); their abundance and form is dictated by specific regional characteristics, such as soil pH (Pérez Corona et al., 1996). The interaction between soil pH and sorption/desorption processes results in these reactions equilibrating with the soil solution (Pierzynski et al., 2005). Research suggests that bioavailable orthophosphate adsorbs to the surface of hydrous metal oxides and clay minerals, through displacement of the OH^- ion or water molecule (Sposito, 1986).

2.1.3. THE PHOSPHORUS CYCLE

Phosphorus is cycled between terrestrial and freshwater systems, in both organic and inorganic fractions. It is controlled by the requirements of the environment and its biota, which results in variation in total phosphorus concentration between different ecosystems with contrasting environmental

pressures. To allow for uptake by their root systems, plants require phosphorus to be held in the bioavailable form, often taking the form of inorganic orthophosphate (Shen et al., 2011). Phosphorus held in non-bioavailable and non-labile species, such as calcium, aluminium or iron phosphates cannot be used by plants or microorganisms, and so require biological or chemical breakdown to bioavailable forms (Adeloju et al., 2016).

As is illustrated in Figure 1, the phosphorus cycle comprises a series of inputs and outputs, linked together through natural and anthropogenic processes. Primary phosphorus minerals form the bulk of the input of phosphorus (Pierzynski et al., 2005), as it is the only naturally-occurring source. Apatite is the most commonly found primary phosphorus mineral in soils, accounting for >95% of phosphorus in the earth's crust (Hallberg, 1992; Jahnke, 1992; Schlesigner, 1991; Stevenson, 1986). As is outlined in Section 2.1.1., apatite minerals are found in a range of species, depending on the other elements in the mineral structure. Chemical weathering of non-bioavailable apatite releases inorganic phosphorus species into the soil solution, which is then cycled between inorganic and organic forms as a result of interaction between natural and anthropogenic processes.

The dominant anthropogenic pathway for phosphorus to enter the soil environment, is the addition of mineral fertilisers to soil. Most mineral fertilisers contain nitrogen, phosphorus and potassium, and so are commonly referred to as NPK fertilisers; these nutrients are most limited in soils and therefore added artificially to boost productivity. The phosphorus in NPK fertiliser originates from primary phosphorus minerals found in rock phosphate, which is crushed and added to fertiliser compounds. Adding phosphorus in the form of mineral fertilisers immediately increases the concentration of phosphorus found in the soil solution (Pierzynski et al., 2005). Initially, the phosphorus is primarily involved with adsorption and precipitation process, both of which produce non-bioavailable phosphorus species; these processes are, initially, easily reversible and so phosphorus can rapidly be made available for uptake by plants or can be lost due to surface runoff and leaching (Pierzynski et al., 2005). Over time, the solid phosphorus forms produced due to adsorption and precipitation processes may be converted to less soluble forms, resulting in bioavailable phosphorus concentrations in soil decreasing further (Pierzynski et al., 2005). Mineral fertilisers must be continually applied to maintain sufficiently high concentrations of phosphorus to ensure soils and plants will not be limited with respect to phosphorus.

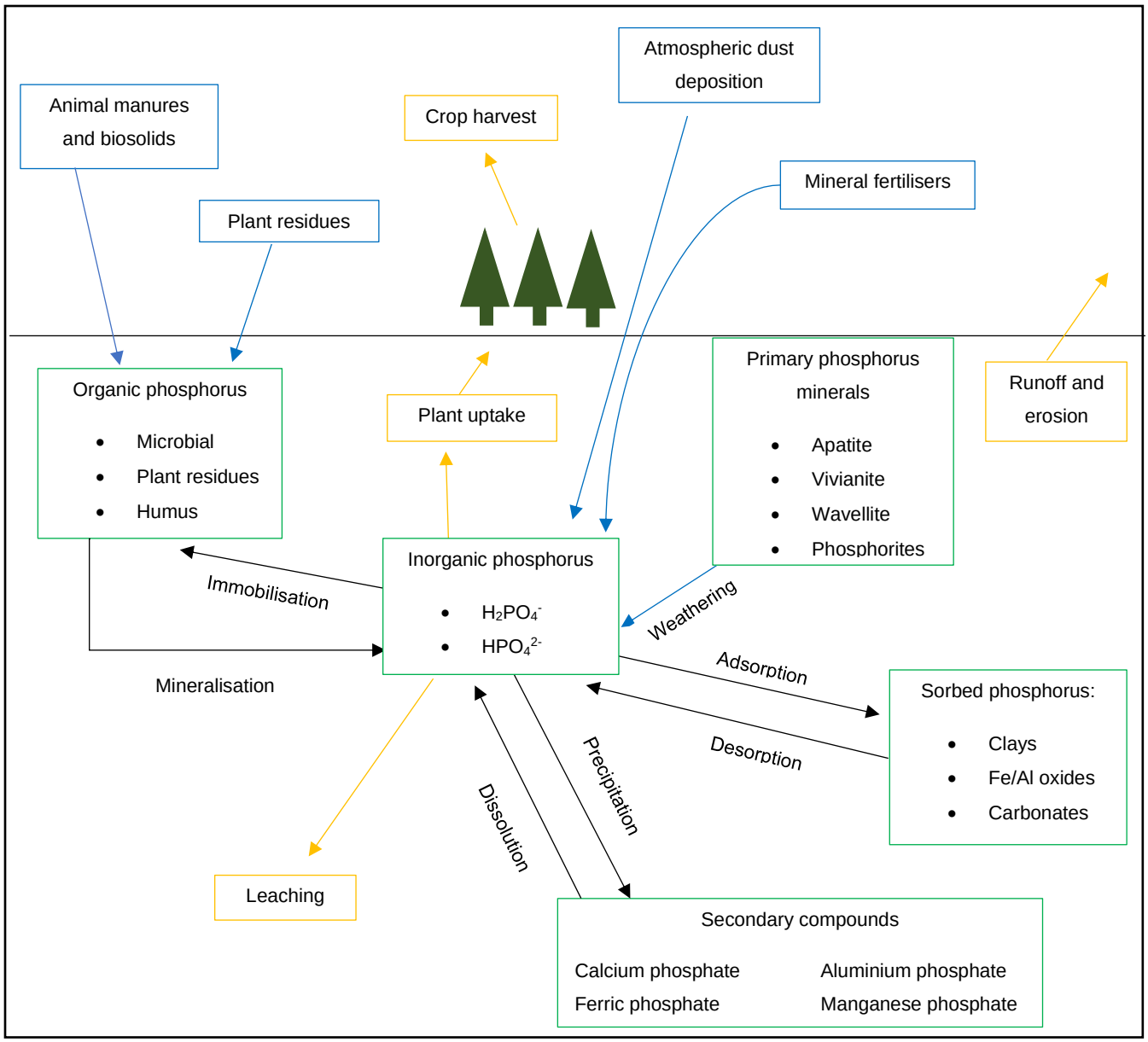


Figure 1 - Schematic of the phosphorus cycle in terrestrial and freshwater environments. Blue outlines show inputs of phosphorus to the terrestrial ecosystem; green outlines show different phosphorus species and yellow outlines show output of phosphorus from the terrestrial ecosystem. Diagram adapted from Celi and Barberis (2005) and Pierzynski et al (2000).

There are further pathways by which phosphorus is added to soil, many of which are naturally-occurring processes influenced by anthropogenic activity. For example, the breakdown of plant residues, animal biosolids and faecal matter can contribute organic phosphorus, which can be accessed by primary producers via mineralisation to produce a bioavailable form of phosphorus; this will be discussed further in this section. The deposition of atmospheric dust, often in the form of wind-blown loess, contributes bioavailable inorganic phosphorus to soils and freshwaters. Loess is a clastic sediment, formed through the accumulation of wind-blown dust; the loess is characterised by a dominance of quartz within its chemical structure, but the composition varies depending on the dust source and other processes

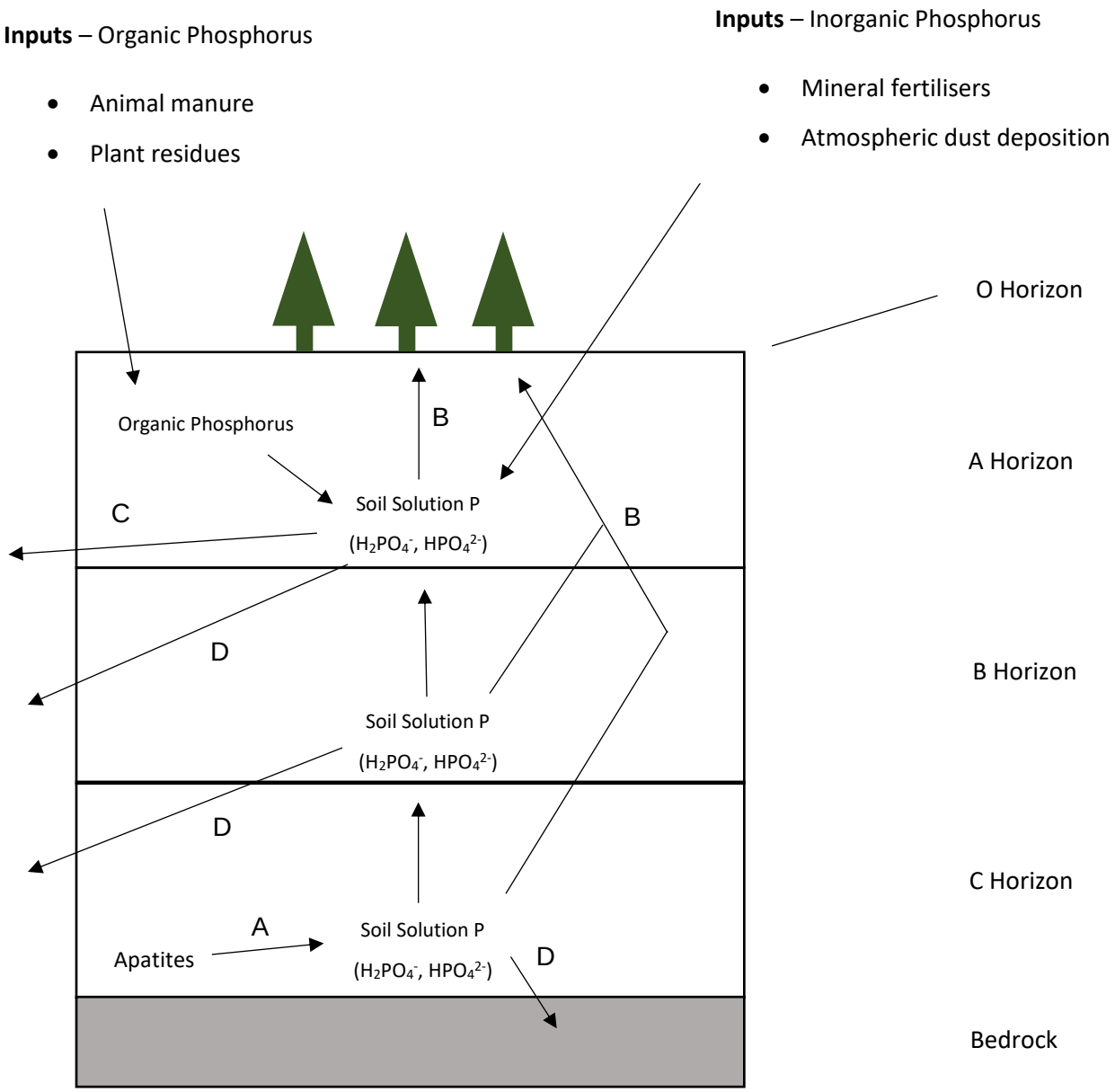


Figure 2 – Schematic of the current understanding of phosphorus cycling between soil horizons (A, B and C). Phosphorus fractions are shown in each soil horizon, in addition to the biogeochemical cycles associated with their transformations:

A: Dissolution ***B: Plant Uptake*** ***C: Runoff and Erosion*** ***D: Leaching***

Data for diagram sourced from: Celi and Barberis (2005); Pierzynski et al (2005) and Ippolito et al (2010).

occurring post-accumulation (Pye, 1995). Although a natural process, wind-blown loess can be influenced by climate-driven desertification or through open-karst mining; both desertification and mining can increase wind-blown dust, which could in turn increase the concentrations of phosphorus, and other elements, present at the deposition site.

In contrast to phosphorus input processes to soils, there are also multiple pathways by which phosphorus is lost from soils. Identified by yellow outlines in Figure 1, phosphorus is either actively removed from soils due to anthropogenic activity or lost via natural processes. Pierzynski et al (2005) argue that crop harvest is the greatest contributor to phosphorus loss from soils, but that runoff and erosion are also significant, and are far more environmentally detrimental given the action of eutrophication. Eutrophication is defined as “the addition of mineral nutrients to an ecosystem, generally raising the net primary productivity” (Simmons, 2000, p. 185), and is usually attributed to sewage effluent or fertiliser runoff from farmland. The impact of eutrophication is often seen in the form of deoxygenation of water as a result of increased bacterial activity and growth, and can occur in freshwater and salt water ecosystems (Foy and Withers, 1995; Simmons, 2000). Through improving understanding of the interactions between fertilisers and the surrounding ecosystem, more targeted methods of fertiliser application could be adopted in mainstream farming practice, subsequently reducing phosphorus loss from soils and better protecting aquatic ecosystems from eutrophication. The final mechanism of phosphorus loss from soils is the process of leaching, whereby soluble nutrients are lost from soils due to rain and excess irrigation. In sandy soils, leaching of nutrients to groundwater is seen as a significant issue, but can be difficult to monitor or trace if there is limited knowledge of the local subsurface geology or hydrology.

Further to the input and output mechanisms for phosphorus in soils, there are several important biochemical processes which control the cycling of phosphorus between different forms. Mineralisation is a key process in phosphorus cycling in soils. It refers to a process where inorganic phosphorus is released from organic phosphorus held in the soil. Inorganic phosphorus can either be released from organic phosphorus components in the soil, or due to the decomposition of plant and microbial matter held in the soil matrix (Condron et al., 2005). Despite significant research into this area, the process of mineralisation is still poorly understood; it is thought that mineralisation rate is controlled by extracellular and periplasmic enzymes (Frossard et al., 2000; Magid et al., 1996; Stewart and Tiessen, 1987). Extracellular enzymes are those produced and secreted by a cell, which performs a function outside of the cell itself; periplasmic enzymes are found to function between the cytoplasmic membrane and the cell envelope, within the periplasmic space (Beacham, 1979). Periplasmic and extracellular enzymes are produced by the plant roots, mycorrhizae and soil microorganisms according to their demand for phosphorus in relation to the availability of soil inorganic phosphorus (Dalai, 1977; McGill and Cole, 1981; Olander and Vitousek, 2000; Sinsabaugh, 1994). Organic phosphorus species can also be broken

down by organic acids, such as citric, lactic and oxalic acid, which are secreted by some soil microorganisms (Louw and Webly, 1959), such as endomycorrhizal and ectomycorrhizal fungi. This process is discussed further in Section 2.3. Agricultural cultivation of soils has been found to enhance mineralisation rates, with previous studies indicating that organic phosphorus concentrations can decrease by 81% in soils that have undergone 60 to 90 years of cultivation (Tiessen et al., 1983). Given the involvement and action of plants and microorganisms in mineralisation, the process is highly influenced by soil temperature and moisture; it is found to be most rapid when soils are warm and moist but well-drained (Pierzynski et al., 2005).

Immobilisation involves the biological conversion of inorganic phosphorus to organic phosphorus via two major pathways (Condron et al., 2005). In the first instance, inorganic phosphorus is removed from the soil solution and associated solid-phase pools; this occurs during the microbial decomposition of organic residues that have a high carbon to phosphorus ratio (>300) (Condron et al., 2005). Some of this phosphorus will be subsequently released as organic phosphorus compounds in detritus following cell death and lysis (Alexander, 1977). Immobilisation also involves inorganic phosphorus, that has been removed from the soil by plant uptake, being returned in the organic fraction in leaf litter and root debris, in addition to animal excrement and manure (Alexander, 1977; Condron et al., 2005). This process can be interrupted and the system balance altered depending on phosphorus fertiliser inputs to the system; the rate of organic phosphorus accumulation in soils declines as equilibrium becomes established between organic phosphorus inputs and mineralisation rates (Condron and Goh, 1989).

Figure 1 highlights several other mechanisms which control the phosphorus cycle, including precipitation of inorganic phosphorus, adsorption of bioavailable phosphorus species and desorption of phosphorus from soils. Precipitation is the process of bioavailable phosphorus reacting with elements in the soil to form phosphate minerals. In acid soils, phosphorus generally binds to iron, aluminium and manganese, whilst it is preferentially bound to calcium in alkaline soils (Pérez Corona et al., 1996). This process forms the basis of phosphorus stripping from sewage treatment, whereby aluminium and iron ions are used to remove excess phosphorus from waters (Cooper et al., 1993; Jenkins et al., 1971). It is a more permanent process than the temporary change in species seen in adsorption and desorption processes. Adsorption and desorption are contrasting mechanisms, which involve the binding of inorganic bioavailable phosphorus to soil particles, rendering it non-bioavailable (Pierzynski et al., 2005). Desorption is the opposite process, whereby inorganic bioavailable phosphorus species are released from their adsorbed state into the soil, increasing the bioavailable phosphorus pool.

The inputs and outputs, in addition to the internal biogeochemical cycles, of the phosphorus cycle are relatively well understood as a result of literature contributing to a wide knowledge base. Finer-scale cycling processes remain very poorly explored and understood, with only a small number of papers

exploring phosphorus cycling in relation to soil horizons. Horizons vary depending on a large number of factors, including organic inputs, climate and underlying bedrock but usually maintain the pattern of an A horizon at the surface, underlain by the B horizon, which in turn is underlain by the C horizon and subsequently the bedrock (Bridges, 1993). Figure 2 highlights this structure and layout of soil horizons, whilst illustrating the current knowledge in relation to phosphorus cycling between soil horizons. It is understood that anthropogenic inputs, such as fertiliser and animal manure, affect phosphorus concentrations in the A horizon most significantly. Uptake of phosphorus by biota is most likely to occur in the A horizon, as a result of root depths and the bioavailability of phosphorus from fertilisers. In highly-degraded environments, where soils may be thin and nutrient-poor, plant roots can extend down into the B and C horizons to uptake phosphorus from the soil solution pools (Ippolito et al., 2010). Phosphorus can also be removed from all horizons via leaching, whilst the A horizon is also at risk of phosphorus loss due to runoff and erosion due to entrainment of phosphorus-rich sediments in overland flow (Pierzynski et al., 2005). Organic and inorganic phosphorus species are known to be inputted at the soil surface (the A horizon); as is shown in Figure 1, there is a good understanding of the biogeochemical processes responsible for transformations of phosphorus species within soils. What remains poorly understood if these cycles are linked to soil horizons, and if so, how these vary across the soil profile. Given the wide range of soil types and associated characteristics, it is likely that these cycles will vary both spatially and temporally, making it a complex area of research to conduct.

The phosphorus cycle, although a collection of natural processes, have been highly influenced by human activity; agriculture is the dominant anthropogenic forcing on the phosphorus cycle, with farming practices increasing both the inputs and outputs of phosphorus in the environment. Soils given over to agricultural practices are often found to have higher total phosphorus concentrations than non-agricultural soils (George et al., 2004), however in most cases, these concentrations of phosphorus are still below the optimum levels required for plant cultivation (Adeloju et al., 2016). To counterbalance the deficit in bioavailable phosphorus in agricultural soils, inorganic phosphorus fertilisers are added to boost total soil phosphorus; although primarily used to ameliorate the bioavailable phosphorus deficit, such fertilisers also increase insoluble, non-bioavailable phosphorus, which raises total soil phosphorus concentrations (Richardson et al., 2001; Sanyal and DeDatta, 1991). Research indicates that only 10-20% of the phosphorus applied in mineral fertilisers is actually bioavailable and therefore able to be used by plants, with the remaining phosphorus converted into non-bioavailable organic and inorganic species (Holford, 1997). Given the effect of fertiliser runoff on aquatic ecosystems, it is key that the management of soil nutrients allows for the optimum concentration of 0.003 to 0.3 mg P L^{-1} to be present within cultivated soils (Pierzynski et al., 2005), whilst preventing the concentration of total P found in surface waters from exceeding 0.03mg P L^{-1}, which has been acknowledged as the threshold for the detrimental eutrophication of freshwaters (Pierzynski et al., 2005). This challenge has given rise to an

ever-growing area of research, exploring methods for increasing phosphorus uptake by plants through methods other than increased surface application of mineral fertilisers.

2.2. PHOSPHORUS AND PLANTS – ROLES AND INTERACTIONS

Like all living organisms, plants require phosphorus as a key nutrient for the storage and reproduction of genetic material, in addition to playing a role in metabolic processes such as growth and cell reproduction; the element forms part of the deoxyribose-phosphate backbone of DNA and RNA, which carry the genetic material for all living organisms (Elser et al., 2007; Smits et al., 2012) Phosphorus is required for growth in primary producers, as it forms much of the structure of cell membranes and proteins; it therefore acts to inhibit primary production when the nutrient is limited in the environment (Hanrahan et al., 2004). In most soils, phosphorus is the main limiting nutrient for primary production, either directly, or indirectly by limiting nitrogen fixation (Vitousek and Howarth, 1991). Phosphorus limitation in soils results in a high demand for mineral fertilisers, which provide bioavailable phosphorus to plants. Phosphorus resources are finite and rapidly-depleting, and given the increase of global phosphorus cycling by 400% since the industrial revolution (Falkowski et al., 2000), greater understanding of the mechanisms used by plants to acquire bioavailable phosphorus is required to ensure the future of sustainable agriculture (Smits et al., 2012; Stutter et al., 2015).

Plants use a variety of mechanisms to augment uptake of phosphorus from soils which are limited by bioavailable phosphorus (Atwell et al., 1980). These mechanisms include alterations to the root system, through development of highly-branched roots, with fine root hairs to increase the surface area of the root system (Richardson et al., 2001). The formation of symbiotic relationships with mycorrhizal fungi is considered to be another mechanism that has been adapted to increase the availability of phosphorus in soils; such fungi exude compounds from roots that can either directly or indirectly increase soil phosphorus availability and uptake (Raghothama, 1999; Richardson, 1994; Richardson et al., 2001; Schachtman et al., 1998). An example of root exudates that indirectly increase phosphorus uptake is the secretion of phosphatase compounds, which are used in mineralisation, to release bioavailable phosphate from organic phosphorus compounds in soils (Raghothama, 1999). The role of mycorrhizal fungi in influencing phosphorus uptake by plants will be discussed further in Section 2.3.

Phosphorus, nitrogen and other nutrient ions are taken up by plants via three processes: root interception, mass flow, and diffusion (Barber, 1984). Root interception refers to the process by which soil nutrients are absorbed when the root comes directly into contact with the nutrient ions. The rate of interception is governed by root volume, and is therefore subsequently influenced by both the plant species and the presence of mycorrhizal fungi, as they act to increase the surface area of the root system (Barber, 1984; Bolan, 1991). Plants also uptake nutrients via mass flow, whereby nutrients are taken up during the absorption of water by a plant; this process is controlled by the volume of water absorbed, and the nutrient concentration in the water (Bolan, 1991). Previous research indicates that ions that are less easily retained by soils, such as nitrate, chloride and sulphate, which are taken up by

mass flow; phosphate and potassium are readily retained by soils, and are therefore less likely to be taken up by plants via mass flow (Barber, 1984; Bolan, 1991). Diffusion of nutrient ions into the root system occurs in the absence of other uptake methods; the uptake of selective nutrients by plant roots creates a concentration gradient between the soil and the root surface. There then occurs net movement of nutrients from soil to the root surface, from an area of high nutrient concentration to low nutrient concentration. Phosphorus uptake by diffusion may be too low to meet the requirements of the plant if soils have a low solubility or high fixation capacity for phosphorus (Gerke, 1992; Hoberg et al., 2005; Sale and Mokwunye, 1993).

It is thought that in the case of phosphorus, diffusion is the dominant process for uptake by roots. Root interception is limited by the volumetric capacity of the roots themselves, as many annual crops have root systems with a volume of <1% of the overall soil volume (Bolan, 1991). This dictates that the uptake of phosphorus will be <1% of available soil phosphorus, which is far less than most plants require (Bolan, 1991). Mass flow is unable to transport adequate levels of phosphorus to plants, given the low concentrations of phosphorus that are found in calcareous soils (Chapin, 1980).

As a result of the very low concentrations of both total and bioavailable phosphorus found in most soils, phosphorus is considered the primary limiting nutrient in almost all cultivated soils. Phosphorus limitation in plants can present itself in a number of ways, including the stunting of growth or poor formation of leaves and shoots (Schertz, 1921). The clearest indication of phosphorus limitation in plants is the presence of purple-brown mottling on the leaves, in addition to purple discolouration of the plant stems and shoots (Schertz, 1921). These symptoms can occur in any plant species, however are most noticeable on plants with green or variegated foliage. In plant species which are naturally have purple or brown mottling, it is important to be able to determine whether the discolouration can be attributed to the species itself, or whether phosphorus limitation is the cause of the colouring.

Previous research concludes that the phosphorus concentration of plant leaves and shoots is equal to the concentration found in plant roots when cultivated in non-nutrient-limited soils (He et al., 2015). However, if soils are limited with respect to phosphorus, it has been found that plant roots tend to have higher phosphorus concentrations than the above-surface plant biomass (Chapin, 1980). It is thought that in phosphorus-limited soils, plants prioritise the growth of additional roots over the growth of further leaves and shoots; this allows for greater exploration of the soil environment, with the intention of acquiring additional phosphorus to prevent nutrient deficiency within the plant (Chapin, 1980; He et al., 2015). Phosphorus has no gaseous phase and can only be taken up through plant roots systems, and therefore increasing the phosphorus uptake potential through expanding the overall surface area of the root system allows for a better chance of plant survival, despite the nutrient-limited soil.

2.3. ECTOMYCORRHIZAL AND ENDOMYCORRHIZAL FUNGI IN PLANTS

One of the oldest and most significant adaptations of plants in order to increase nutrient uptake is the formation of symbiotic relationships between mycorrhizal fungi in soils and plant roots (Smith and Paul, 1990); such associations occur in over 80% of plant species (Smith and Read, 2008; Wang et al., 2010). Mycorrhizal fungi are found in most soils (Abbott and Robson, 1982; Jasper et al., 1989) and fall into two distinct categories: ectomycorrhizae and endomycorrhizae. Ectomycorrhizae (ECM) are characterised by mycelium sheaths around plant root systems, and are mainly found in temperate forest conditions (Bolan, 1991); in contrast, endomycorrhizae (EDM) form external networks of hyphae within the soil matrix, and are associated with most plant species (Bolan, 1991). The most commonly found EDM species are the vesicular-arbuscular mycorrhizae (Bolan, 1991).

Evidence suggests that mycorrhizal fungi enhance the uptake of phosphorus by plants from soils where concentrations of bioavailable inorganic phosphorus are low (Abbott and Robson, 1982; Mosse, 1973; Tinker, 1978). In soils that contain limited bioavailable phosphorus, it is thought that mycorrhizae have the greatest impact in increasing the amount of phosphorus that is accessible to the plant (Hetrick, 1989). Bolan (1991) outlines the different mechanisms used by mycorrhizal fungi to enhance the uptake of phosphorus by primary producers. Mycorrhizae increase the length and surface area of the root system (Rhodes and Gerdemann, 1975; Tinker, 1978), which in turn increases the volume of soil that can be accessed by the plant; this makes "positionally unavailable nutrients available" (Bolan, 1991, p. 194). Further to this, the increased root length and surface area helps to shorten the diffusion distance between soil phosphorus and the plant, therefore augmenting the phosphorus uptake rate (Sanders and Tinker, 1973); mycorrhizal fungi are thought to double the absorption surface area for an individual plant (Gianinazzi-Pearson and Gianinazzi, 1983). In addition to an overall increase in root surface area, the development of mycorrhizal hyphae allows for access into smaller soil pores, which would be inaccessible by root hairs alone (Bjorkmann, 1949).

In addition to increased exploration of the soil, it is thought that mycorrhizae cause greater movement of phosphorus into the plant roots. When examined in relation to non-mycorrhizal plants, mycorrhizae-infected roots have been found to absorb more phosphorus, suggesting mycorrhizal hyphae have a higher affinity for phosphate ions (Bolan, 1991; Bolan et al., 1987; Cress et al., 1979). However, this is contested within the research community, with Karunaratne, Baker and Barker (1986) observing no such increased affinity for phosphorus within mycorrhizal roots. They concluded that infection of plant roots by mycorrhizae results in an increase in the number of uptake sites per unit area of plant root. Although no firm conclusions have been drawn about the specific action of mycorrhizal roots, there is evidence that the presence of mycorrhizal fungi results in an overall increase in plant growth (Hayman, 1983; Hetrick, 1989; Menge, 1983; Mosse, 1973). The current research struggles to quantify the increase in

growth that can be seen in mycorrhizal species (Hayman, 1983). Much of the research to date has used sterilised soils, which subsequently fails to consider the impact of the microflora of the soil, in addition to the influence of symbiotic relationships between multiple plant species found within the natural environment (Ames et al., 1984; Hetrick, 1989; Meyer, J.R., Lindermann, 1986).

Mycorrhizal fungi also modify the rhizosphere to increase phosphorus uptake, through a range of active and passive methods (Bolan, 1991). ECM fungi exude hydrogen ions, chelating compounds and phosphatase enzymes, which have a solubilising effect on poorly-soluble phosphorus compounds such as apatite (Allen et al., 1981; Routien and Dawson, 1943). Such interactions between ECM fungi and mineral phosphorus are vital in ensuring soil solution phosphorus in nutrient-limited soils does not become severely depleted. Previous research has identified that mycorrhizal fungi increase phosphorus availability in soils through the solubilisation of inorganic phosphorus species or by the mineralisation of organic phosphorus (Bolan, 1991; Hetrick, 1989). Seeling and Zasoski (1993) found that this biological action is a significant factor in the solubilisation of organic phosphorus; when microbial action is removed through autoclaving or sterilisation of soils, organic phosphorus becomes undetectable in leachate water despite being the largest soluble phosphorus compound found in leachate from non-autoclaved or unsterilised soils (Condron et al., 2005; Seeling and Zasoski, 1993). Such findings indicate that mycorrhizal fungi are killed off during the sterilisation or autoclaving process, disabling them from interacting with phosphorus held in soils. This is considered to be a main limiting factor in laboratory-based research in this field, given that most studies use sterilised or autoclaved soil samples in their experimental research; often, this is often due to licencing constraints enforced by regulatory bodies such as DEFRA, in relation to cultivating imported soils.

Plant roots and mycorrhizal fungi exude low molecular weight organic acids (LMWOAs) such as citrate, malate and oxalate to modify the rhizosphere and increase nutrient uptake (Jones, 1998; Yadav and Tarafdar, 2003). LMWOAs have been found to influence the solubility and subsequent mineralisation rates of organic phosphorus within the rhizosphere environment (Chen et al., 2002; Fox and Comerford, 1990; Hayes et al., 2000; Herbert and Bertsch, 1995). Previous research highlights a relationship between the presence and activity of mycorrhizal fungi, and a lowering of pH; Bonneville et al (2011) concluded that mycorrhizae cause significant acidification around plant roots, which in turn increases apatite dissolution rates. Depending on soil type, concentration and speciation of organic acid, it is thought that root exudation of LMWOAs such as malate, oxalate and citrate can lead to soil solution phosphorus concentrations that are 10 to 1000-fold greater than normal (Fox et al., 1990a, 1990b; Fox and Comerford, 1992; Jones and Darrah, 1994).

There are a wide range of LMWOAs present within plant root systems, including oxalate, citrate and malate (Fox and Comerford, 1990; Froidevaux, L., Kälin, 1981; Jones, 1998; Routien and Dawson,

1943). These acids are able to complex metal ions in solution, and so cause the dissolution of soil minerals (Huang and Keller, 1972; Jones and Kochian, 1996; Pohlman and McColl, 1984); organic acids are able to cause a 2 to 4 fold increase in metal ion dissolution rate when compared with rainwater (Li et al., 2012). Oxalic acid is of particular interest, given it precipitates in the presence of Ca^{2+}, which may be important when considering phosphorus release from calcium enriched minerals such as the apatite minerals (Jones, 1998; Li et al., 2012). Research conducted by Panhwar et al (2013) concluded that significantly higher rates of phosphorus uptake were seen in plants inoculated with phosphate-solubilising bacteria and additional treatments of organic acids, in particular the addition of oxalic acid. Oxalic and malic acid treatments were added to aerobic rice plants inoculated with phosphate-solubilising bacteria (PSB) at concentrations of 0, 10, 20, 30 mM. Significantly higher concentrations of solubilised phosphorus of 31.5% were found in PSB-inoculated aerobic rice which had been treated with 20mM of oxalic acid (Panhwar et al., 2013).

It is also important to consider how these findings from Panhwar et al (2013) are transferable to research using other soil types. Panhwar et al conducted research using Christmas Island Rock Phosphate, which comprises of the amorphous residues of calcium, aluminium and iron phosphates; the chemical composition of this rock phosphate is very different to previously-studied apatite compounds (Khasawneh and Doll, 1979; Palmer and Gilkes, 1983). Calcium-rich soils and sediments have a higher buffering capacity for LMWOAs, and therefore higher concentrations of LMWOAs would often need to be applied to produce the same chelating effect upon phosphorus compounds within the soil structure. Mobilisation of phosphorus from soils using LMWOAs is dependent not only on acid concentration, but also on a complex range of interactions including: diffusion rates, microbial degradation and sorption and desorption reactions (Cline et al., 1987; Jones and Darrah, 1994). Given the complexity of the acid-phosphorus compound interactions, determining suitable LMWOA concentrations for soil treatments can be challenging and must include consideration of the soil type and composition.

2.4. SPECTRA AND THE KARST CRITICAL ZONE OF SOUTH WEST CHINA

Figure 3 - Chenqi catchment in 2016, showing the range of land-use types found within the catchment. In the foreground of the photo, there are signs of arable crop cultivation, with primary and secondary forest on the steeper hilly areas. Image c/o Dr Sophie Green, University of Exeter.

Figure 4 - Landscape degradation in Chenqi catchment. The images highlight the desertification across the Chenqi subcatchment, including exposed bedrock and thin soils. Images c/o Dr Sophie Green, University of Exeter.

2.4.1 GLOBAL KARST ENVIRONMENTS

Estimated to cover between 7% and 12% of Earth's continental area (Hartmann et al., 2014), karst environments are characterised as landscapes that are shaped by erosion and chemical weathering of carbonate bedrock such as limestone or gypsum. Weathering processes result in complex subsurface drainage systems comprising of caves and sinkholes (Bull, 2005; Park and Allaby, 2013). Initially, all karst regions have active surface drainage controlled by fluvial systems; over time, this develops into a subsurface system whereby water is circulated through an underground network of caves and voids (Dreybrodt, 1988; Weary and Doctor, 2014). The specific dynamics of a subsurface drainage system is dependent upon how the regional geology has been shaped by tectonic activity (Song, 1999).

The dynamic nature of karst geology and topography results in fluctuating soil depths; as is seen in Figure 5, thin, nutrient-poor soils are common where the bedrock is located close to the surface, whilst deeper soils are found in fissures and depressions in the bedrock (Bull, 2005; Song, 1999). Often, karst regions are dominated by thin soils, due to the significant deforestation that is employed to increase land area suitable for crop cultivation (Song, 1999); deforestation often leads to increased soil erosion and reduced vegetation cover, resulting in a highly degraded, desertified environment, that could take many years to recover and re-vegetate (Yuan, 2001).

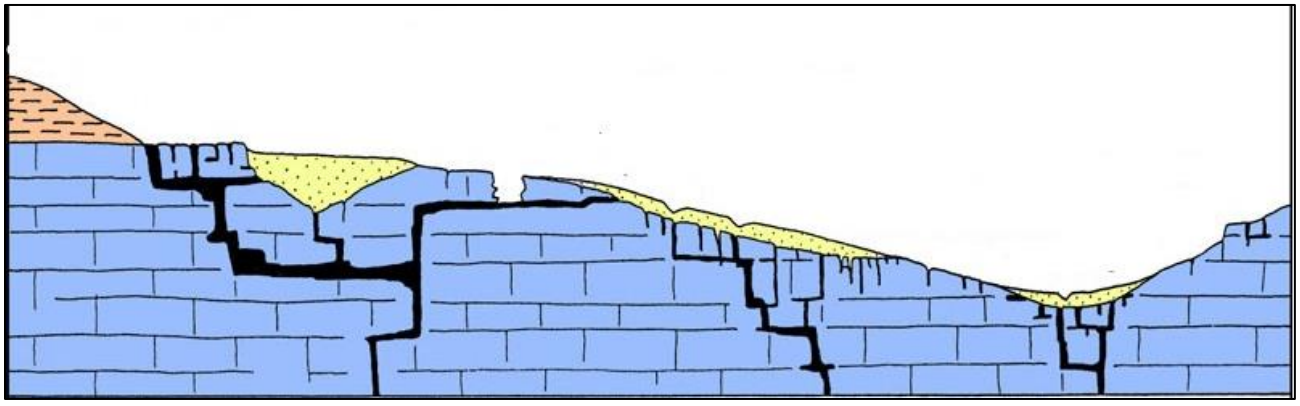


Figure 5 - Cross-section of a mature karst system, highlighting the variation in soil depths across a karst landscape. Soils are shown as yellow dotted areas, whilst blue blocked areas represent the underlying limestone. Adapted from Waltham and Fookes (2005).

Phosphorus, in terms of both total and soluble phosphorus, is very limited in highly weathered soils, such as those that characterise karst environments (Kertesz and Frossard, 2015). Phosphorus is highly immobile in calcareous soils (Niinemets and Kull, 2005), and is known to be the primary limiting factor for plant growth in karst environments (Jones, 1998; Tyler, 1992). The storage of nutrients, particularly that of phosphorus, is recognised as an important factor in aiding vegetation recovery in desertified karst regions (Du et al., 2011; Hofmeister et al., 2002; Piao et al., 2005; Rivera et al., 2010). This is due to calcareous soils with high pH being deficient in bioavailable phosphorus rather than nitrogen (Du et al.,

2011; Hu et al., 2009; Liu et al., 2006; Niinemets and Kull, 2005). Previous studies have found low-molecular-weight organic acids in "appreciable quantities" of 0.0001 – 0.1µmol g^{-1} (Li et al., 2012, p. 195) in soils; in karst environments this may augment the dissolution of inorganic phosphorus phases, enhancing bioavailable phosphorus availability in the soil solution pool (Li et al., 2012).

To counteract the shallow depths and poor nutritional content of karst soils, farmer often use nitrogen, phosphorus and potassium (NPK) fertilisers to promote crop production; although this boosts crop yields in many karst environments, it presents issues that threaten the hydrological and ecological stability of the karst. Soil erosion and overland flow leads to transportation of fertilisers into the water course or subsurface drainage system, subsequently polluting the groundwater (Ford and Williams, 2007). Groundwater pollution in karst environments is a prevalent issue, as karst aquifers are known to provide at least a partial source of potable water to a quarter of the global population (Ford and Williams, 2007). Karst aquifers, like all groundwater resources, are at risk of eutrophication through the over-fertilisation of arable land by NPK fertilisers and other anthropogenic actions. Increased understanding of nutrient cycling in karst environment is required, to allow for introduction and management of sustainable agricultural practices.

2.4.2 THE KARST CRITICAL ZONE OF SOUTH WEST CHINA

Subtropical karst terrain accounts for 0.34 million km^2 in south west China, with the region subject to significant desertification (Yuan, 2001). Thin and nutrient-poor soils, coupled with large rocky outcrops leads to limited primary production and reduced vegetation cover (Bull, 2005; Wei et al., 2011; Yuan, 2001) as is seen in Figure 6**.** Depending on specific regional characteristics and anthropogenic influences, highly degraded environments such as the south China karst can take many years to recover and re-vegetate. The desertification and landscape degradation experienced in south west China is caused by deforestation, overgrazing and intensified crop cultivation, which has subsequently influenced the region's ecological and hydrological regimes (Liu et al., 2006; Yuan, 2001).

73.6% of Guizhou Province lies within the highly-degraded south China karst (Critical Zone Exploration Network, 2016). The province is somewhat unusual, in that unlike other karst regions across the world, it has a relatively high, and ever-increasing population density (Baiping et al., 2006); as of 2016, the province has a total population of 35 million. Much of the karst region of south west China is used for agriculture, in particular the cultivation of arable crops; commonly-grown crops include soybeans, maize and rice, which are grown in rotation to reduce nutrient stripping from soils (University of Exeter, 2017). The agricultural landscape in the karst critical zone is generally confined to the base of the valleys and the surrounding lower hillslopes, where soils are comparatively deeper and less prone to erosive action. Given the poor nutrient concentrations found in the karst soils in Guizhou, NPK fertilisers are known to

be regularly applied by farmers to improved total crop yield. Data from the Chinese government indicates that NPK fertiliser application is increasing at a rapid rate, with an increase of 160 10^5 tons per year over the last 20 years. In 1996, 320 10^5 tons of fertiliser was applied to land across southwest China, and by 2016 this figure measured 480 10^5 tons (Green et al., *in press*).

With a population of 35 million people, Guizhou Province is of particular threat in relation to exhaustion of nutrients, poor crop yield and subsequent soil erosion. The large population generates further challenges that must be considered when developing methods for karst management and cultivation. With such a large population that relies so heavily upon subsistence agriculture, it is not possible to restrict farming in Guizhou as a method for restoring the landscape. Instead, methods must be adapted to consider the needs of those who live within the karst critical zone, whilst also safeguarding the soil and its nutrients for future generations. The rebuilding of the karst ecosystem is of relative urgency, given the reliance upon the landscape for agricultural production and subsequent economic development (Wei et al., 2005, 2011; Zhu et al., 2006). Studies have previously attempted to establish methods for restoration of the south China karst, in particular in examining the characteristics of soil microbes in karst regions regions (Yu et al., 2002). However, there remains a poor understanding of the relationship between microbial activity in karst soils and vegetation recovery. It is hoped that the research conducted within the SPECTRA project will provide a better understanding of how nutrient-limited karst regions, such as that found in Guizhou Province, can be better managed to improve ecosystem recovery and resilience.

Figure 6 - Maize crops growing in Chenqi subcatchment in 2016. Crops are grown across all available land-surfaces, despite the thin soil depths and exposed outcrops of rock. Image c/o Dr Sophie Green, University of Exeter.

2.4.3. SPECTRA: SOIL PROCESSES AND ECOLOGICAL SERVICES IN THE KARST CRITICAL ZONE OF SOUTH WEST CHINA

This research falls within the SPECTRA project, an international NERC-Newton-Funded project, that is investigating soil processes and ecological services within the karst critical zone of south west China. The project is a collaboration between researchers at a range of institutions in the UK and China:

UK: University of Exeter; University of Bristol; Cranfield University; Rothamstead Research

China: Chinese Academy of Sciences; Tianjin University; Peking University; Beijing Normal University

A crucial focus of the SPECTRA project is to examine the response, resilience and recovery of the south China karst to natural and anthropogenic perturbations (University of Exeter, 2017). It is hoped that findings from the research can be used to enhance the sustainable development of Guizhou, one of the most economically-deprived provinces in China. 73.6% of Guizhou is classified as karst landscape (Baiping et al., 2006), and with a population of 35 million (2016), it has been recognised as a region which has significant socio-economic pressures, further exacerbated by the highly-degraded landscape. The project is focusing upon the geological, hydrological and ecological processes in the south China karst, and how these interact and control the soil fertility and function within the landscape. It is thought that improved understanding of the interactions between these processes will allow for the introduction of management techniques that help maximise the delivery of ecosystem services across south west China (University of Exeter, 2017).

2.5. PLANT GROWTH EXPERIMENTS

A significant limitation of soil-based research is the lack of experiments that have been conducted on native, unsterilised soils (Hetrick, 1989). The physical and chemical impact of soil autoclaving is poorly explored but is known to impact upon microflora likely present in the soil; soil microflora is thought to have a significant impact upon plant nutrient uptake (Hayman, 1983; Hetrick, 1989). The removal of soil microflora by sterilisation limits research, by failing to consider the role of inter- and intra-specific competition in plant communities (Hetrick, 1989). The primary reason for using sterilised soils in laboratory-based research is due to the guidelines and constraints enforced by regulatory bodies such as DEFRA in the UK. Soils imported from outside of Europe require an import licence, and to be labelled as biohazardous material, resulting in a requirement for specific laboratory conditions if the soils are to be used experimentally without prior sterilisation.

A suite of experiments were conducted by Sainz and Arines (1988) to explore the forms of phosphorus taken up by plants inoculated with mycorrhizal fungi in comparison to the species of phosphorus obtained by uninfected plants. The experiments were based on the principle of phosphorus fractionation, whereby a sequential extraction of phosphorus was conducted on the soils prior to cultivation, and again conducted once the plants have matured (Sainz and Arines, 1988). The difference in concentrations of different phosphorus fractions provided an insight into the forms of phosphorus acquired by plants, and how this differed when mycorrhizal fungi had been used to infect plant roots (Bolan, 1991). In their research, Sainz and Arines (Sainz and Arines, 1988) concluded plant species that were inoculated with mycorrhizal fungi, and those that remained uninfected both used the same source of inorganic phosphorus within the soil profile. In recent years, there has been little work that uses such a method to determine phosphorus uptake by plants; this may be due to the highly specific nature of the conclusions that can be drawn. The uptake of phosphorus will be specific to both the plant species chosen and the mycorrhizal fungi used for inoculation; furthermore, there are complications in relation to phosphorus cycling and leaching, making it difficult to determine the exact interactions between different forms of phosphorus within the soil environment.

Further to exploring the influence of mycorrhizal fungi upon the uptake of phosphorus by primary producers, experimental sequences have been used to better understand the influence of autoclaving or sterilisation of soils on the availability of phosphorus in the soil solution. Through estimation of algal growth rather than through plant-based experiments, (Anderson and Magdoff, 2005) conclude that autoclaving of soils results in approximately 60% more bioavailable phosphorus than in non-autoclaved samples of soil. This research also found that the autoclaving process resulted in concentrations of soluble phosphorus up to 78% higher than in non-autoclaved soils. Anderson and Magdoff (2005) concluded that the changes in soluble phosphorus concentrations in autoclaved soils was as a result of

the increases in both temperature and pressure that occur in autoclaving. It is though that the high pressure and temperature converted phosphorus held in complex organic compounds into smaller bioavailable organic subunits (Anderson and Magdoff, 2005). The autoclaving process is also thought to break down phosphorus held in microbial cells into inorganic orthophosphate, which is also a soluble, bioavailable fraction (Anderson and Magdoff, 2005). The research conducted by Anderson and Magdoff (2005), although conclusive, was based on algal growth on soils rather than plant growth; there currently remains a gap in the available literature that targets the effect of autoclaving on phosphorus availability for uptake by different plant species.

In many previously-conducted plant-based experiments, which aim to quantify the growth of plants in a specific growth medium, a specific methodology is chosen to attribute the results to the nutrient concentration of the soil. In research conducted by Zhang et al (1997), which aimed to quantify the impact of LMWOAs exuded from root systems on the uptake of phosphorus in a specific growth medium, the seedlings were germinated in a quartz-based sand before repotting into the growing medium. This ensured that healthy seedlings were being transplanted into the growing medium, and so any change in the condition of the plants could likely be linked to deficiencies in the growth solution. Although growth solutions are not being used in this research project, the same method of transplanting healthy seedlings into experimental soils could be used, to ensure that any changes to the plant growth can be attributed to nutrient deficiencies in the soil, rather than unsatisfactory germination that could be associated to a range of issues such as unsuitable environmental conditions or simply a genetic issue with the seeds.

3.0. RESEARCH AIMS AND OBJECTIVES

3.1. RESEARCH AIMS

To investigate, using experimental methods, the role of oxalic acid exudates from mycorrhizal fungi in influencing the uptake of phosphorus from phosphorus-limited soils from the karst critical zone of south west China.

3.2. RESEARCH OBJECTIVES

To examine, using scanning-electron-microscope analysis, the species of phosphorus found within calcareous soils from the karst critical zone of south west China.

To better understand the role of acid exudates from vesicular-arbuscular mycorrhizae in the uptake of nutrients from soil.

To examine the influence of organic acids in the breakdown of non-bioavailable phosphorus, and the subsequent mobilisation of bioavailable species.

3.3. RESEARCH QUESTION

Is phosphorus uptake by primary producers in calcareous soils from karst regions controlled by organic acid exudates from mycorrhizal fungi?

4.0. HYPOTHESES

Based upon the state of the knowledge displayed in the current literature, a number of hypotheses were formed, which apply to the research conducted within this project.

1. Soil samples collected from the karst critical zone of south west China will be limited with respect to phosphorus.
    a. Plants grown in untreated soils collected from the karst critical zone of south west China will indicate signs of phosphorus limitation.
    b. SEM EDS analysis of soil samples from south west China will show low phosphorus abundance.

2. Plants grown in soils treated with oxalic acid will have a greater total plant biomass than plants grown in untreated soils.

3. Plants grown in soils treated with oxalic acid will not present symptoms of phosphorus limitation.

4. Plants grown in soils treated with the highest concentration of oxalic acid (40mM) will have a greater total biomass than plants grown in soils treated with 20mM or 0mM oxalic acid (Panhwar et al., 2013).

5. Total phosphorus concentration in plant roots will be greater than the total phosphorus concentration of plant leaves and shoots (Chapin, 1980).

5.0. METHODOLOGY

5.1. SITE SELECTION

Guizhou Province, as is highlighted in Figure 7**,** within the karst landscape of south west China, is one of the most severely degraded and desertified environments in the world. Subtropical karst covers 0.34 million km^2 across south west China (Yuan, 2001), and accounts for 73.6% of the total area of Guizhou Province (Baiping et al., 2006). Karst environments are formed in regions underlain by calcium-rich rock such as limestone, gypsum or dolomite; such regions often experience high annual rainfall, which dissolves the relatively-soluble bedrock, forming the sinkholes and caves that are indicative of karst landscapes (Bull, 2005).

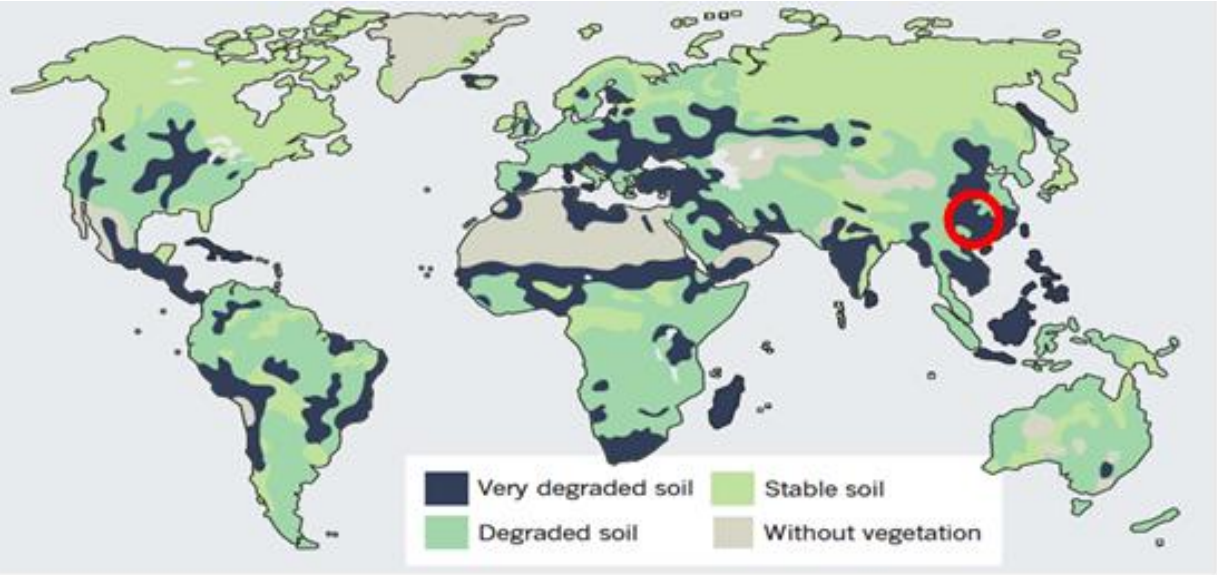


Figure 7 - Global soil degradation state, including very degraded regions which are often associated with karst environments. Red ring shows location of Guizhou Province. Image taken from Banwart (2011).

In Guizhou Province, desertification is caused by a number of anthropogenically-driven factors, including deforestation, overgrazing and intensified arable crop cultivation, which has subsequently impacted upon the region's ecological and hydrological regimes (Liu et al., 2006; Yuan, 2001)**.** Guizhou has a population of 35 million people, resulting in significant anthropogenic pressure being placed upon the karst environment as a resource for subsistence farming and socio-economic growth. These pressures must be considered when preparing management and policy for restoring degraded karst regions such as are located across Guizhou Province.

Figure 8 - Location of Chenqi subcatchment, within Guizhou province. Image obtained from Google Earth LANDSAT.

Chenqi catchment (26°15'49.6188"N, 105°46'18.9696"E) , located in Puding County, Guizhou Province is one of the primary study areas for the work conducted within the SPECTRA research project (Critical Zone Exploration Network, 2016). Highlighted in Figure 8, Chenqi covers 1.29 km^2 of karst landscape, with a catchment elevation of 1310 – 1470 m above sea level (Critical Zone Exploration Network, 2016). As is seen in Figure 3, Chenqi catchment contains both primary and secondary forest; primary forest refers to "naturally regenerated forest of native species, where there are no clear visible indications of human activities and the ecological processes are not significantly disturbed" (FAO, 2012, p. 7). Secondary forest are "forests regenerating largely through natural processes after significant removal or disturbance of original forest vegetation by human or natural causes at a single point in time or over an extended period" (Chokkalingam and Jong, 2001, p. 21). Primary forest is cleared to make way for subsistence farming; in Chenqi, some of the deforested land is eventually recolonised as secondary forest, whilst the primary farmland that is too severely degraded becomes abandoned farmland. This is characterised by large rocky outcrops, where soils have become too thin for crop cultivation to occur, as is seen in Figure 4.

There are two further dominant land-use types found in Chenqi subcatchment: cultivated farmland and abandoned farmland. Much of the farmland is currently being cultivated, to meet the needs of the local population, growing maize, rice and soybeans as the main crops. These crops are grown in rotation, in

part to reduce nutrient stripping from the soils. Over time, as a result of farming practices, soil erosion and the nature of karst environments, the soil in areas given over to farming become too thin and nutrient-poor to be used successfully for arable cultivation. These areas are then abandoned and tend to “re-wild” with native species. Over time, the lack of intensive agriculture tends to improve the nutrient concentrations found in the soil, in addition to creating deeper soils through allowing plant biomass to rot back into the soil rather than being removed, as occurs in intensive agricultural practices. In the long term, these areas of abandoned farmland could once again be considered for active cultivation, however at present, the area remains abandoned and unable to be cultivated.

5.2. DEFRA LICENSING AND SOIL AUTOCLAVING

The soil samples collected from Chenqi catchment in 2016 were classified as biohazardous material and were therefore held under DEFRA licence PHL 103615/198219/4. In accordance with the licence, all soils were autoclaved prior to use in any experimental work; the licence states that all samples must be autoclaved prior to plant life being grown on them, unless there is access to a laboratory that has clearance for cultivating plants on licenced soils.

Autoclaving is the process of sterilisation by high temperature and high-pressure steam, with the aim of inhibiting microbial activity within the soils (Razavi darbar and Lakzian, 2007; Shaw et al., 1999; Trevors, 1996). In accordance with the DEFRA licence, soils collected from Chenqi catchment must be autoclaved at 121°C and 15 pounds per square inch (PSI) for a minimum of 30 minutes, to become registered as unlicensed material that is free of microbial activity. All autoclave bags must be marked using autoclave tape prior to being autoclaved; this tape must be checked after the autoclaving process is complete. In accordance with the DEFRA licence, any autoclave tape that has not darkened after undergoing an autoclave cycle should be run again in the autoclave, to ensure thorough sterilisation.

5.3. PETROGRAPHIC THIN SECTIONS

Petrographic sections are thin sections of rock, soil or bone that can be analysed using a petrographic microscope or scanning electron microscope (SEM). Samples are usually impregnated within epoxy resin, before being ground and polished to expose crystals for analysis. SEM analysis can produce an elemental map of the sample; this is a microscopic image that reveals the size, shape and spatial distribution of different elements within the sample (Goldstein et al., 2017). Elemental analysis of a sample can also be conducted using energy-dispersive X-ray spectroscopy (EDS), which is able to characterise the chemical composition of a sample (Goldstein et al., 2017).

In this research project, SEM imaging and SEM EDS analysis will allow for elemental identification of the forms of phosphorus present in the soil samples from Chenqi. SEM EDS analysis will be used to highlight the chemical composition of any phosphorus present in the Chenqi samples; this analysis technique can identify the chemical elements bound to phosphorus-containing species in the soil. It can also be used to highlight the distribution of phosphorus species across the soil horizons, in addition to the density to which it is found in the soil matrix.

Petrographic thin sections of the karst soils were created using autoclaved soil samples taken from a soil pit in an area of abandoned farmland in the Chenqi subcatchment. Samples of soil from the A horizon, soil rock and rock-soil interface were autoclaved at 121°C for 30 minutes, before being dried at 40°C for 48 hours, to become listed as unlicensed material. The soil samples collected from Chenqi in 2016 have a high clay content, which results in high water retention; therefore, the soils were dried in a drying cabinet at 40°C for 48 hours, to prevent soil moisture from inhibiting complete impregnation by epoxy resin.

5.3.1. MOUNTING THIN SECTIONS

Epofix™ cold-setting embedding resin was used to impregnate the soil samples. Epofix™ resin uses a two-part system, comprising of resin and hardener; this resin was chosen due to its low viscosity and ability to penetrate the low porosity soil samples. The Epofix™ is mixed by volume, in a ratio of 15 parts resin to 2 parts hardener. The epoxy must be mixed for 3-5 minutes prior to use, to produce the best results for impregnation; the resin must be left to stand for 5 minutes after mixing, to allow any bubbles to evacuate prior to use.

Aluminium sample rings were sealed to a glad slide using double-sided tape, and a small sample of the autoclaved soil arranged using tweezers. A thin layer of mixed epoxy resin was used to cover the soil samples. As little epoxy as possible should be used, to increase the chance of air successfully evacuating the sample, therefore producing the highest quality thin section. A needle was used to press

down the soil particles, to ensure that they were properly attached to the tape; the needle can also be used to pop bubbles held within the resin matrix. Vacuum impregnation is required to effectively produce petrographic thin sections using highly porous samples, such as the soils collected from Chenqi. Using a vacuum to impregnate the samples ensures that any air trapped within the soils is evacuated, allowing the resin to better penetrate the sample. This should produce a thin section that will better withstand the grinding and polishing stages of sample preparation. The samples were run in the vacuum for 5 minutes, to allow for the evacuation of air; samples were then removed from the vacuum and left to set for 24 hours. The thin sections were then removed from the glass slides using a razor blade, before undergoing the grinding and polishing process.

5.3.2. GRINDING

Grinding is used to remove the top layer of tape adhesive and epoxy resin from the thin section, to expose the impregnated crystals prior to being polished. Four grades of grinding paper are used, ranging from 240 to 1200; the coarsest paper (240) is used first, working up to the 1200 for the final grinding.

Deionised water was used on the grinding paper for lubrication, whilst the thin section mount was placed into a mount holder, to aid the grinding process. Times allocated for each stage of grinding are highlighted in Table 2**.** Between each stage of grinding, the samples were cleaned with deionised water and allowed to dry thoroughly.

Table 2 - Recommended time period required for grinding petrographic thin sections using different grades of grinding paper. Information taken from School of Earth Sciences, University of Bristol.

Grinding Paper	Time (minutes)
240	3
400	5
600	8
1200	10

After each stage of grinding, the sample surface must be examined using a microscope, to ensure that the soil samples were not scratched, and there were no deep grooves or lesions in the epoxy. Any such faults in the resin can make it difficult to conduct thorough analysis using the SEM.

5.3.3. POLISHING

In the production of petrographic thin sections, polishing is used as the final stage to prepare the sections for analysis using the SEM. An automatic polisher was used, with a sequence of diamond polishing cloths ranging from 9µM to 1µM. Before polishing, and between each change in polishing cloth grade, the thin sections must be cleaned in a beaker of deionised water in an ultrasonic bath for five minutes, to remove any larger grade particles that could contaminate the polishing cloths or damage the thin section itself. The sequence of diamond polishing cloths are used for varying lengths of time, as is outlined in Table 3**.**

Table 3 - Recommended time period required for polishing petrographic thin sections using different polishing cloths. Actual time used is included, as polishing times were adjusted based on the fragility of the samples. Information taken from School of Earth Sciences, University of Bristol.

Polishing Cloth (µM)	Recommended time (minutes)	Time used (minutes)
9	5-10	5
3	30-45	40
1	5	5

To produce the best polished finish on such soft samples as those collected from Chenqi, the automatic polisher was set to the minimum pressure of 5 N (H. Goodes, personal communication, 31st May 2018). Conditions were set on the automatic polisher to shorten the polishing time, so as to further preserve the sample; the head and plate were set to rotate in opposite directions, which shorten the polish time, in addition to reducing pressure on the sample as a whole. MetaDi lubricant was used, in addition to the polishing compounds, to provide lubrication between the thin section ring and the polishing plate. After polishing, the sections were carbon-coated using a carbon-evaporator; this is to avoid the effects of charged particles on the sample surface, and to produce a higher-quality elemental map using SEM EDS analysis.

5.3.4. SEM EDS ANALYSIS

SEM EDS analysis sees the examination of the elemental composition of a rock, soil or bone sample using X-ray analysis at very high resolutions, producing in-depth images which highlight surface topography. For the sections made using Chenqi soils, SEM imaging and SEM EDS analysis methods were run, to produce elemental spectrum data and SEM images of the soil samples. An elemental

spectrum of each soil horizon was produced, which provides a breakdown of the occurrence of different elements within the sample. For each soil horizon, SEM maps were produced, to identify the presence of different elements within the soil; iron and aluminium were chosen for identification and analysis, given that iron-bound and aluminium-bound species of phosphorus are commonplace in many soils. This was identified through research of the current literature in Section 2.1.1.

Due to low porosity of the samples, only small areas of the soils in each section were fully impregnated by the epoxy resin, meaning only small areas of the section were viable for analysis. Using the SEM, the whole section was visually examined, and the largest least damaged area was chosen for analysis. It was not deemed necessary to analyse each cluster of soil individually, given the amount of time that SEM analysis takes and the comparatively small part this analysis plays within the overall research project.

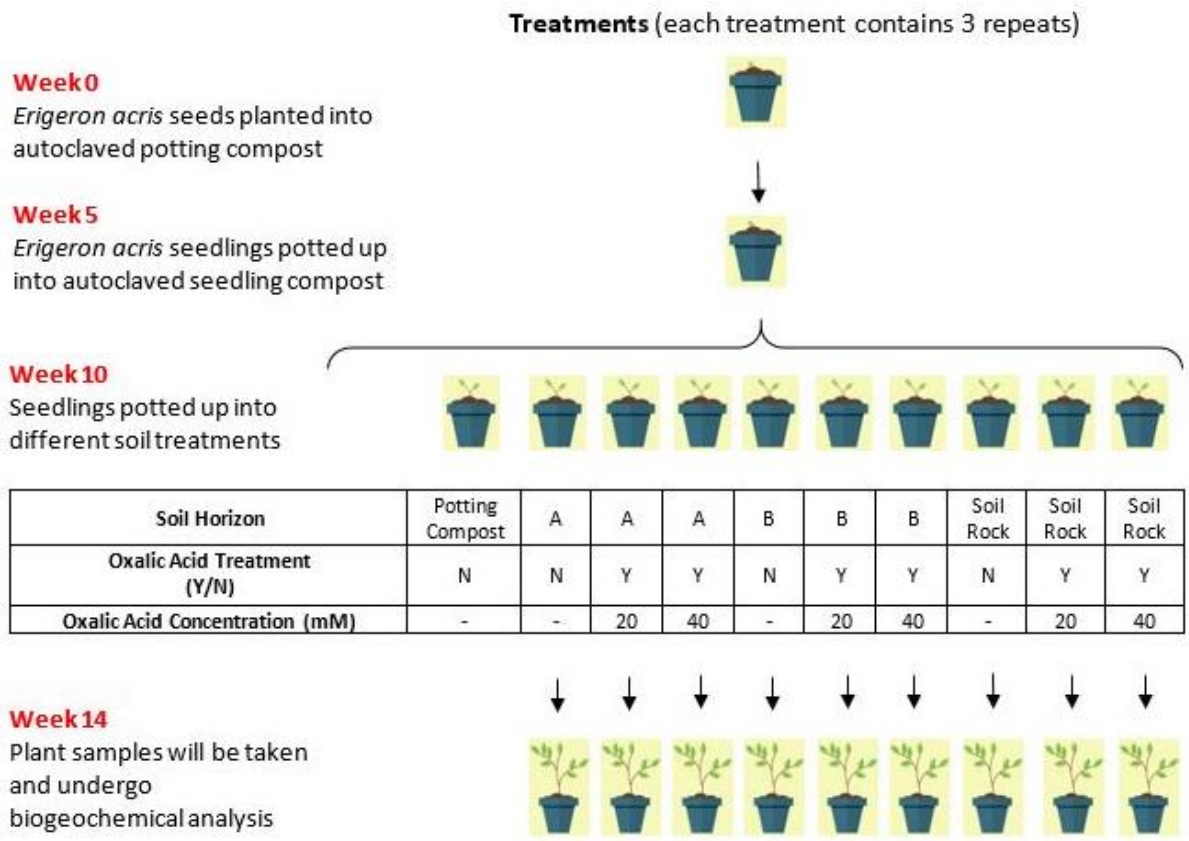
5.4. PLANT EXPERIMENTS

A sequence of plant growth experiments were designed, to better understand the relationship between soil phosphorus content, oxalic acid exudates and phosphorus uptake by primary producers. *Erigeron acris* was chosen for the experiments, based on information obtained through the SPECTRA project. The Chenqi subcatchment was surveyed by SPECTRA researchers in 2016, to identify all plant species growing within the study site (J. Dungait, personal communication, October 2017). In accordance with the DEFRA licence assigned to the Chenqi soils, plants grown on the soils must grow wild in the UK, and not be considered an invasive species. The list of plant species in the understory of Chenqi was cross-referenced with information obtained from the RHS to identify species suitable for growth in lab-based experiments. *Erigeron acris,* a member of the daisy family, was considered the most suitable test plant, given its short growth period and its widespread presence across the UK.

As is shown in Figure 9, the experiments began with growing *Erigeron acris* seeds in seed compost, before being potted on into Chenqi soils once the seedlings have grown and stabilised. This method was chosen, as it would allow for better understanding of the overall plant growth within karstic soils; by conducting germination and the initial growth to occur in autoclaved seed compost, before potting on, it allows for the influence of the nutrient-poor Chenqi soils to be identified. If seeds had been planted initially into the Chinese soils, and failed to grow, then it would not have been possible to attribute the lack of growth to specific factors. It could have been that the mycorrhizal fungi in the soils had been killed off in the autoclaving process, that there is no bioavailable phosphorus, or that the growth conditions themselves were unfavourable, and so the seeds would never have germinated. This method of planting and replanting, when coupled with rigorous monitoring of conditions and plant growth, generate a better understanding of the uptake of phosphorus by primary producers in karstic soils.

Due to the nature of the DEFRA licence assigned to the Chenqi soils held at the University of Bristol, all soil samples were autoclaved prior to being used for cultivation of *Erigeron acris.* Autoclaving was carried out using the method outlined in Section 5.2**.** Non-autoclaved soils could only be used for experimental research in a licensed facility; such a licence is not held for the University of Bristol, and so all soils were autoclaved prior to use. The experimental design was altered to accommodate this requirement of the licence, and so rather than investigating the natural action of the mycorrhizal fungi in the Chenqi soils, organic acids were used in some soil treatments to replicate the action of the organic acids exuded by mycorrhizal fungi. There is uncertainty as to whether the mycorrhizal fungi originally found to have colonised the soil will still be present, given that the soils have remained in storage since 2016, when they were collected from Chenqi subcatchment. The seed compost and potting compost used for the initial germination process, and as a growth medium for the control samples was also

autoclaved prior to planting, to ensure than mycorrhizal fungi from external sources were not introduced to the roots of the *Erigeron acris* seedlings.



Soil Horizon	Potting Compost	A	A	A	B	B	B	Soil Rock	Soil Rock	Soil Rock
Oxalic Acid Treatment (Y/N)	N	N	Y	Y	N	Y	Y	N	Y	Y
Oxalic Acid Concentration (mM)	-	-	20	40	-	20	40	-	20	40

Figure 9 - Experimental design for plant growth experiments, including outline of oxalic acid treatments. potting compost was used as a control experiment for 10-14 weeks to ensure the maintenance of suitable growing conditions.

Once autoclaved, the seed compost was dried at 40°C for 12 hours, to remove excess moisture acquired in the autoclaving process. Seed plug trays were filled with autoclaved seed compost, and 4-6 seeds sown per cell. A thin layer of soil was added on top of the seeds, and the trays were watered using tap water. As shown in Figure 10, propagator lids were placed over the trays, to limit evapotranspiration from the plants and evaporation from the soils, further to maintaining a more stable temperature; the trays were placed under growth lamps that remained on for 12 hours in each 24 hour period, running from 7am to 7pm.

Environmental factors were monitored throughout the growth period:

- Temperature – the laboratory was regulated at a temperature of 15-25C for the duration of the experiments.
- Solar radiation – a plant growth lamp was used to regulate the incoming solar radiation for the plants. Plants were placed 50cm below the growth lamp.
- Soil moisture – soils were checked every day to ensure that they did not dry out or had become waterlogged. Given the high clay content of the Chenqi soils, a spray bottle was often used to water the plants, to prevent the soils becoming waterlogged.

The seeds were germinated and left to grow for 5 weeks, before reaching a sufficient size to be potted up into larger seed trays. Seedlings were potted into potting compost, and any very small seedlings, or those that had failed to grow properly were discarded. This step was included to ensure that when seedlings were planted into the Chenqi soils, they were all of a similar size and stage of growth. Given the size and stability of the seedlings, the propagator lids were removed at this stage, but the plants remained under the growth lamp for 12 hours of each 24-hour period.

Figure 10 - Experimental setup for plant experiments. Plants trays were covered by propagator lids and situated under growth lamps in a temperature-controlled laboratory.

After 8 weeks of growth, the seedlings became stable enough to be potted on into the Chenqi soils, as can be seen in Figure 11. The Chenqi soils were prepared using different organic acid treatments; oxalic acid was selected as the most appropriate organic acid to be used in the experimental sequence, given evidence found in the current literature as to the effectiveness of oxalic acid in similar experiments, such as Panhwar et al (2013).

Samples from the A, B and C horizons were collected from Chenqi during a site visit in 2016. These horizons were chosen for this suite of experiments to identify whether a specific soil horizon was a

greater source of phosphorus to plants and biota than others. These samples were selected and analysed separately to help understand the issues surrounding legacy phosphorus from fertiliser applications. As outlined in Section 5.1., much of the Chenqi subcatchment was subjected to intensive agricultural cultivation with regular applications of NPK fertilisers. Through sampling of the A, B and C horizons it allows for examination of phosphorus in soil samples, in terms of both legacy and naturally-occurring compounds. Biogeochemical cycling processes, such as leaching, could be identified through examination of different soil horizons, in addition to the impact of the underlying bedrock on phosphorus concentrations up the soil profile. In the Chenqi subcatchment, the A horizon sample measures from 0 – 23 cm depth, the B horizon from 24 – 32 cm and the C horizon from 33 – 52 cm depth; these relatively shallow depths indicate the very thin nature of the soils found in this highly-degraded karst region.

As shown in

Table 4, oxalic acid was applied at concentrations of 20 and 40mM, in addition to seedlings being planted into soils with no oxalic acid added; soils were treated with acid and left for 5 days, as is commonplace in experiments using organic acids in soils, such as Panhwar et al (2013) . This gave time for the acids to react with any phosphorus present in the soil, in addition to rebalancing the pH of the soil, thus preventing damage to the seedlings through highly acidic soil pH. Oxalic acid concentrations of 20mM and 40mM were chosen for this suite of experiments, in addition to a set of plants grown in untreated Chenqi soils, which were recorded as 0mM oxalic acid. These concentrations were chosen based on the data presented by Panhwar et al (2013), where concentrations of 0 – 30mM of oxalic and malic acids were investigated. This study found that 20mM was the optimum concentration of oxalic acid for dosing soils, in order to increase phosphorus uptake by plants; therefore 20mM oxalic acid was selected for this experiment, in addition to a higher concentration of 40mM. The karstic nature of the soils collected from Chenqi means that they have a high buffering capacity, due to the presence of a calcium-carbonate rich bedrock. This high buffering capacity meant that a higher concentration of oxalic acid was also selected for these experiments, to examine if higher concentrations of oxalic acid were more effective in highly buffered soils. Further increases in oxalic acid could not be tested in this experimental suite, given the volume of soil available for experiments and need for experimental repeats.

Soil moisture data for each horizon was recorded when the soil samples were collected in 2016; this data, displayed in Table 5, coupled with the mass of soil in each pot, was used to calculate the appropriate volume of oxalic acid that should be added to each soil sample. Soil moisture data was recorded for each soil horizon when the samples were collected from Chenqi in 2016. This data, displayed in Table 5, coupled with the mass of soil required to fill each individual pot, was used to

calculate the appropriate volume of oxalic acid to be added to each soil sample. This method was chosen, in order to restore the soil to the same soil moisture as was found during sample collection; ensuring that experimental soil moisture was matched to field recordings of soil moisture was important in preventing soils becoming waterlogged. The Chenqi soils have a very high clay percentage, and therefore are poorly-draining soils; if soils had been dosed with acid in addition to being rehydrated with deionised water, it is likely that the plants would have become waterlogged and growth and health would have been detrimentally affected. For those soils being treated with 0mM of oxalic acid, deionised water was used in place of the oxalic acid to restore soil moisture, to ensure that there was continuity in soil moisture between different samples. To prevent cross-contamination of soils by varying acid concentrations, all pots receiving the oxalic acid concentration were grouped in the same plant trays. This would prevent any leached acid being taken up by other plants or absorbed into other soil samples.

Figure 11 - Erigeron acris seedlings after 8 weeks of growth, prior to potting up into the Chenqi soils that have undergone the treatments outlined in Table 4.

Table 4 - Soil treatments for experimental sequence, including the concentration of oxalic acid that was applied to specific soils.

Soil Horizon	**Potting Compost**	**A**	**A**	**A**	**B**	**B**	**B**	**C**	**C**	**C**
Oxalic Acid Treatment (Yes/No)	N	N	Y	Y	N	Y	Y	N	Y	Y
Oxalic Acid Concentration (mM)	-	-	20	40	-	20	40	-	20	40
Number of Replicates	3	3	3	3	3	3	3	3	3	3

Code	C	0A	20A	40A	0B	20B	40B	0C	20C	40C

Table 5 - Soil moisture values for A horizon, Rock Soil and Soil-Rock Interface for samples collected from Chenqi in 2016.

Soil Horizon	Soil Moisture (%)
A Horizon	32.4
B – Soil Rock	34.1
C – Rock-Soil Interface	37.4

In the process of repotting the seedlings from the potting compost into the Chenqi soils, excess potting compost had to be removed from the roots. This was to ensure that once transplanted into the Chenqi soils, the *Erigeron acris* would be solely using the nutrients within the Chenqi soils. The roots of the *Erigeron acris* were very delicate at the time of repotting, and so it was decided the soil could not be washed out of the roots, as it could cause additional damage to the root systems. Therefore, it was decided that the potting compost should be removed by hand from the roots. Plants were not watered for 3-4 days prior to reporting, to ensure the soils were dry and would be more easily removed from the roots. As much soil was removed by hand as possible, before a brush was used to remove the remaining soil where possible. The seedlings were selected for the repotting based on their size and stage of growth; although having been cultivated for the same length of time, there was distinct variation in size between the seedlings. Many of the seedlings were of a very similar size, with a small number of small and large “outlier” seedlings. These “outliers” were discarded, and those of a similar size chosen for potting up, as can be seen in Figure 12.

Figure 12 - Erigeron acris seedlings potted up into the Chenqi soils. Seedlings have been grouped according to the oxalic acid concentration applied to the soils, to prevent cross contamination.

Once the soil was removed from the roots, each seedling was weighed, and the mass recorded, to allow for plant biomass change before and after cultivation in the Chenqi soils to be examined. Each plant was labelled, using a code system that provides information on oxalic acid concentration, soil horizon and number of repeats. The *Erigeron acris* were replanted into the Chenqi soil, and then watered and placed back into the same growing conditions as previously mentioned, including the use of growth lamps; propagator lids were no longer required, due to the stability of the seedlings. The control seedlings were also subjected to the same method and treatment, whereby they were transplanted from potting compost to potting compost. Their mass was recorded prior to transplanting into the new potting compost, so that biomass measurements could also be analysed for the control samples.

The seedlings were left to grow for 5 weeks, before reaching a sufficient size to undergo the proposed biogeochemical analysis. At this point, a final examination of the plant health and growth quality was undertaken, to determine any visual signs of phosphorus deficiency in the seedlings, such as purple discolouration of the stems or purple-brown mottling of the plant leaves. The final biomass of each plant was also recorded, with all soil removed via rinsing in deionised water prior to being weighed. The *Erigeron acris* were then prepared for acid digestion, as is outlined in Section 5.5.2.

5.5. BIOGEOCHEMICAL ANALYSIS

5.5.1 PLANT HEALTH AND GROWTH QUALITY

Throughout the growing process, the plants were examined for overall health and growth quality, to determine the impact of nutrient-limited soils on plant quality. Phosphorus limitation in soils can manifest itself as visible signs on plant leaves and stems; purple or red-brown mottling on plant leaves and shoots is an indicator of a limitation of phosphorus in the plant biomass, which can in turn cause stunted growth of the plant.

For the *Erigeron acris* grown in this experimental sequence, plants were examined multiple times a week for signs of phosphorus limitation, and any visible signs were recorded and photographed. A control sample of *Erigeron acris* were grown alongside the samples cultivated in Chenqi soils; these control plants were grown in potting compost and were compared to the plants in Chenqi soils to assess the plant health and overall quality of growth.

5.5.2. PLANT BIOMASS CHANGE

The change in total plant biomass was analysed through recording the mass of each seedling before and after cultivation in Chenqi soils. Before planting into the Chenqi soil samples, the whole seedling was weighed, with all soil brushed from the roots. At the end of the growing period, the seedlings were removed from the Chenqi soils, and the roots washed thoroughly to remove all soil traces. Seedlings were individually weighed, including the leaves, shoots and roots, and the difference calculated as a proportion of the initial mass, to determine the percentage change in mass for each seedling. This method of analysis was chosen, as it removed the requirement for all the seedlings to have the same mass prior to being planted in the Chenqi soils.

5.5.3. DIGESTION OF PLANT SAMPLES

Total phosphorus within the plant roots, leaves and stems of the *Erigeron acris* was examined quantitatively, using the *Gallery Plus*, an automated photometric analyser. At the end of the growing term, after the mass of the seedlings had been recorded, the subsurface biomass was separated from the leaves and shoots, to allow for the root matter to be analysed separately. The plant biomass was then placed in a drying cabinet at 80° C for 48 hours, to remove all moisture in preparation for grinding. The dried samples were ground to a fine powder in preparation for undergoing acid digestion.

A wet acid digestion method was used to extract phosphorus from the dried plant biomass, in preparation for the samples to undergo total phosphorus analysis on the *Gallery Plus.* The wet digestion method was taken and adapted from Allen (Allen, 1989), and is commonly used for preparation of plant or soil samples to undergo total phosphorus or total nitrogen analysis. The method is based around a heated digestion using an acid-based reagent; the digestion regent is made up using the following method.

Sulphuric acid-hydrogen peroxide procedure (Allen, 1989)

Reagents:

1. Sulphuric acid (concentrated)
2. Hydrogen peroxide (30%)
3. Selenium powder
4. Lithium sulphate (monohydrate)

Add 0.42g of selenium powder and 14g of lithium sulphate to 350ml of 30% hydrogen peroxide and mix well. Slowly add 420ml of concentrated sulphuric acid whilst cooling the mixture. The reagent must then be stored at 2°C and will remain stable for 4 weeks.

To conduct the wet digestion of the plant matter using the sulphuric acid-hydrogen peroxide reagent, the following method was observed:

1. Weigh 0.2g ± 0.001g of finely-ground plant material or soil into a 100ml conical flask.
2. In a fume cupboard, add 4.4ml of the digestion reagent to each flask, and begin to heat at 200°C on a hot plate. Heat at 200°C for 45 minutes, before increasing the temperature to 360°C for 3 hours. All flasks must be covered by a watch glass throughout the digestion process.
3. 1 hour after increasing the temperature to 360°C, add an additional 4.4ml of reagent to each digestion vessel. Return to the hotplate and allow to digest for another 2 hours, or until the digested matter has turned a sandy-brown colour.
4. Remove the flasks from the hotplate and allow to cool fully.
5. Add 50ml of deionised water to each flask and allow to stand.
6. Using Whatman 42 filter papers, filter the samples into 100ml volumetric flasks.
7. Add enough deionised water to the volumetric flasks to make each sample up to 100ml. Invert the flasks several times to ensure it is well mixed, then decant into labelled sample tubes.

5.5.4. *GALLERY PLUS* ANALYSIS OF DIGESTED PLANT MATTER

Total phosphorus of the digested plant biomass was analysed using the *Gallery Plus* automated photometric analyser. Initially, the samples were run using the “Phosphorus High” test, for which the *Gallery Plus* was calibrated using a phosphorus standard. The calibration data is displayed in Figure 13, which was accepted due to the R-squared value of 0.9987, demonstrating a strong correlation between the phosphorus standard and the associated absorbance values. The digested plant samples were first analysed using the “Phosphorus High” test, however on examination, the data showed that a number of the digested plant samples contained concentraitons of phosphorus too low to be recognised using the “Phosphorus High” test. The samples were then run again on the *Gallery Plus,* using the “Phosphorus Low” test, to try to quantify samples with much lower concentrations of phosphorus. Again, the *Gallery Plus* was calibrated using a phosphorus standard, with the calibration curve for “Phosphorus Low” shown in Figure 14. An R-squared value of 0.9989 was calculated for the calibration data for “Phosphorus Low”, which was accepted before the digested plant biomass samples were run on the *Gallery Plus.* By running all the samples through both the “Phosphorus High” and “Phosphorus Low” test on the *Gallery Plus*, it was possible to capture quantitative results for plant biomass samples containing high or extremely low concentrations of total phosphorus.

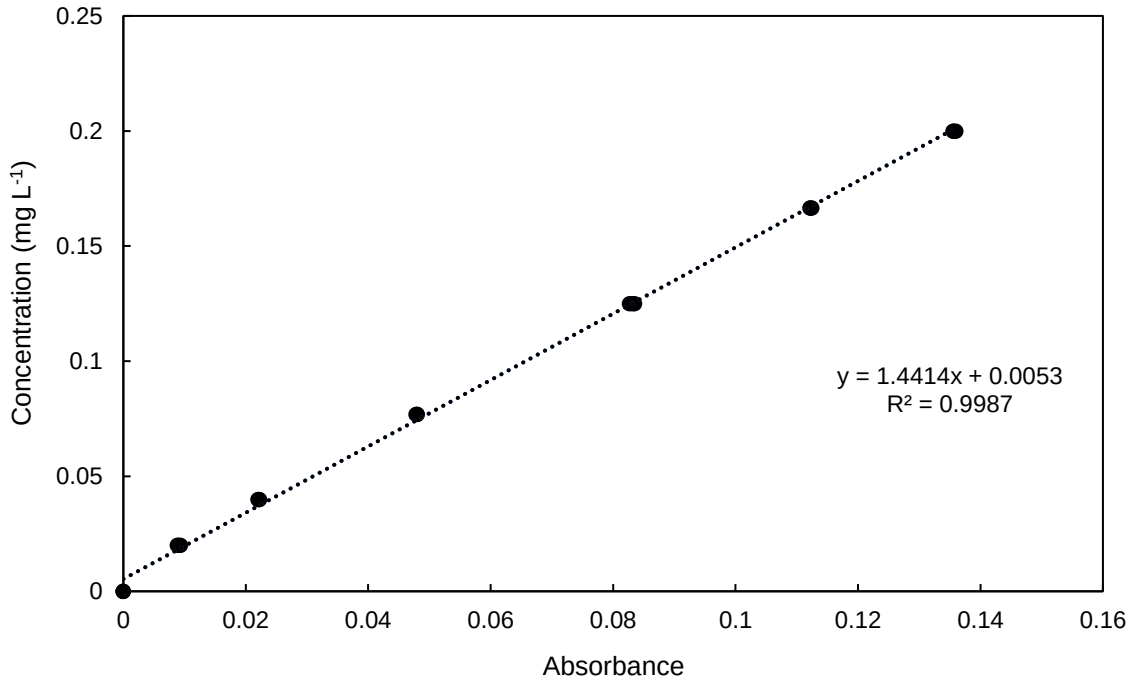


Figure 13 - Calibration curve for "Phosphorus High" test on the Gallery Plus automated photometric autoanalyser. An R-squared value of 0.9987 was generated for the calibration data and was therefore accepted prior to analysing the digested plant biomass samples.

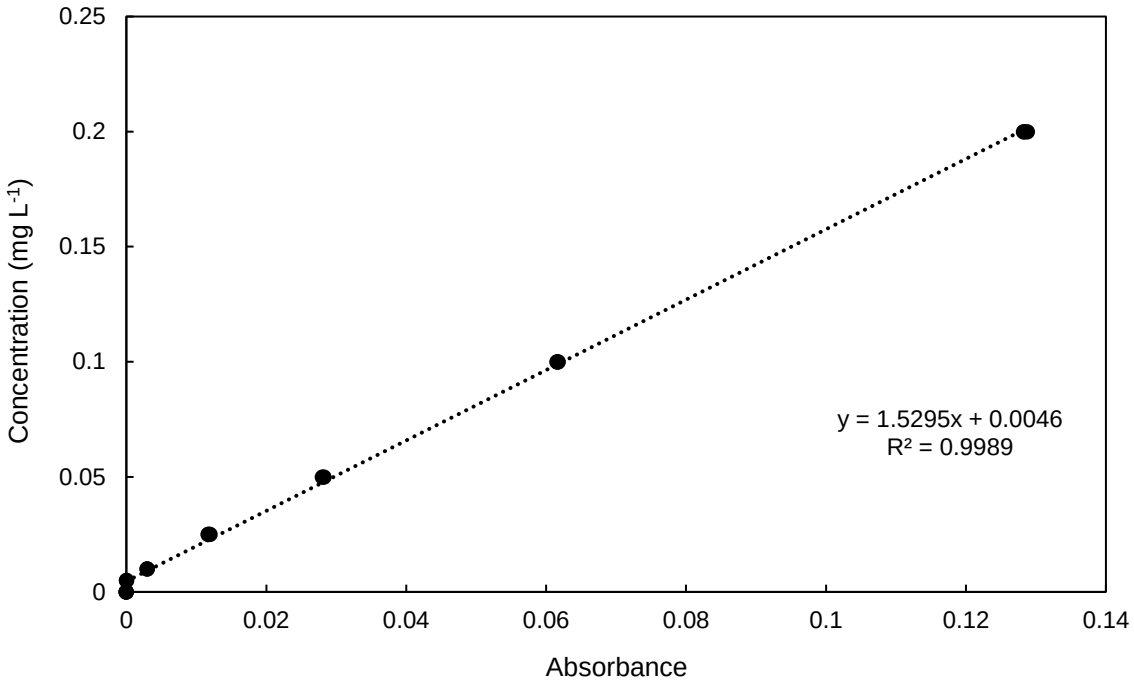


Figure 14 - Calibration curve for "Phosphorus Low" test using the Gallery Plus automated photometric autoanalyser. An R-squared value of 0.9989 was calculated for the calibration data and was therefore accepted prior to running the "Phosphorus Low" analysis on the plant biomass samples.

5.5.3. SAMPLE CODES

A coding system was used to refer to the samples undergoing biogeochemical analysis using the *Gallery Plus* automated photometric autoanalyser. The coding system provides information referring to the soil horizon, oxalic acid concentration, repeat number and whether the sample was taken from the above-ground plant (leaves and stems) or the roots. Figure 15 shows how sample codes have been assigned to each of the samples run through biogeochemical analysis methods. Note that a full list of sample codes and related characteristics can be found in Table A1 in Appendix A.

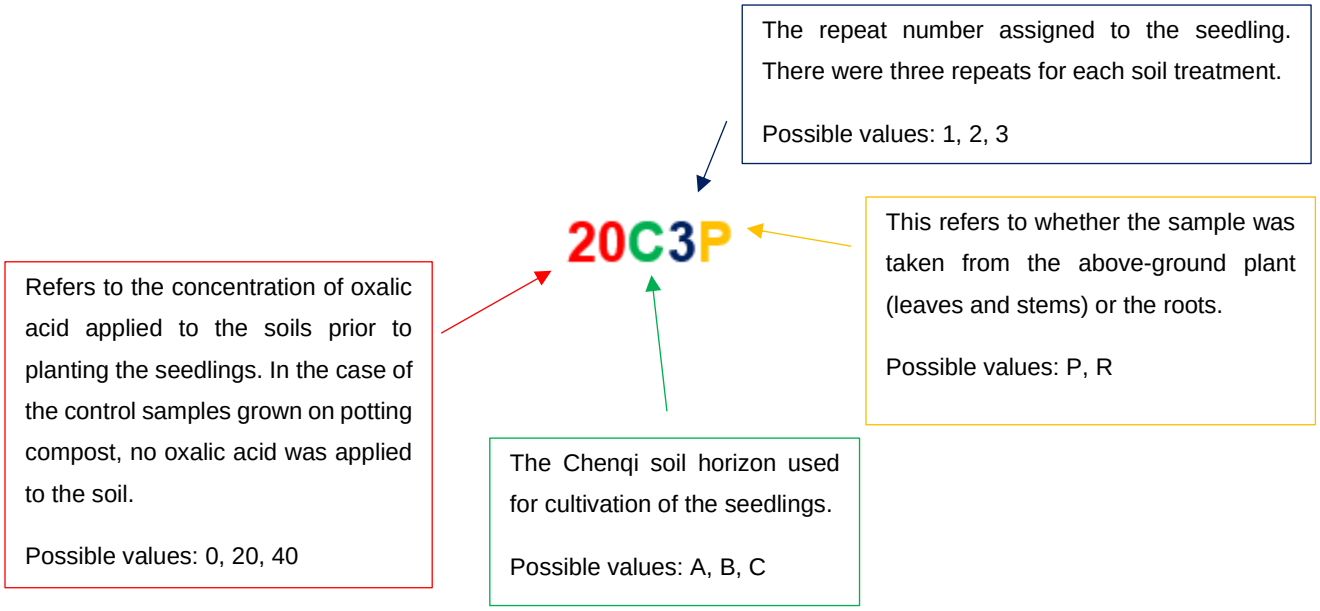


Figure 15 - System for assigning sample codes. The sample codes will be used throughout this work to refer to specific samples and groups of samples with similar characteristics. A comprehensive list of all sample codes and characteristics can be found in Appendix A.

6.0. RESULTS

6.1. SEM EDS IMAGING AND ANALYSIS

SEM EDS analysis was used in this research to produce spectrum data and elemental maps for each of the soil horizons collected in Chenqi. Spectrum data was generated for each of the horizons, whilst elemental maps were produced for a range of elements within each of the soil horizons. The elemental maps use colour to highlight the presence of a specific element or elements in a sample; the lighter an area of the elemental map, the higher the concentration of the specified element. Elemental maps can also be layered using the SEM EDS software, allowing for the user to identify if there are multiple elements present in one area of the sample; for example, layering phosphorus and iron elemental maps can be used to identify if ferric phosphate compounds are present in the sample. As with single element analysis, the lighter coloured areas of the map show a higher concentration of the compounds.

6.1.1. A HORIZON

Figure 16 illustrates the spectrum data that was generated from the SEM EDS analysis of the A horizon sample taken from Chenqi in 2016. The data highlights that the soil composition is dominated by several elements, including silicon, oxygen, aluminium, titanium and manganese. The spectral analysis does not indicate anything above background concentrations of phosphorus, as would be expected in such a phosphorus-limited karst region. The dominant binding for phosphorus in the soils of this region is with iron as ferric phosphate, as is indicated by the data presented in Appendix B. Given the low levels of iron identified in the spectral analysis, it is unlikely that there will be high concentrations of phosphorus present in the A horizon soil.

The following section displays results of SEM EDS analysis on A horizon soils. Several elements were analysed using SEM EDS, however there is a primary focus on phosphorus, calcium, iron and aluminium, given the prior knowledge of phosphorus binding that is laid out in the current literature. Titanium and manganese was also analysed, and this data can be seen in Appendix C.

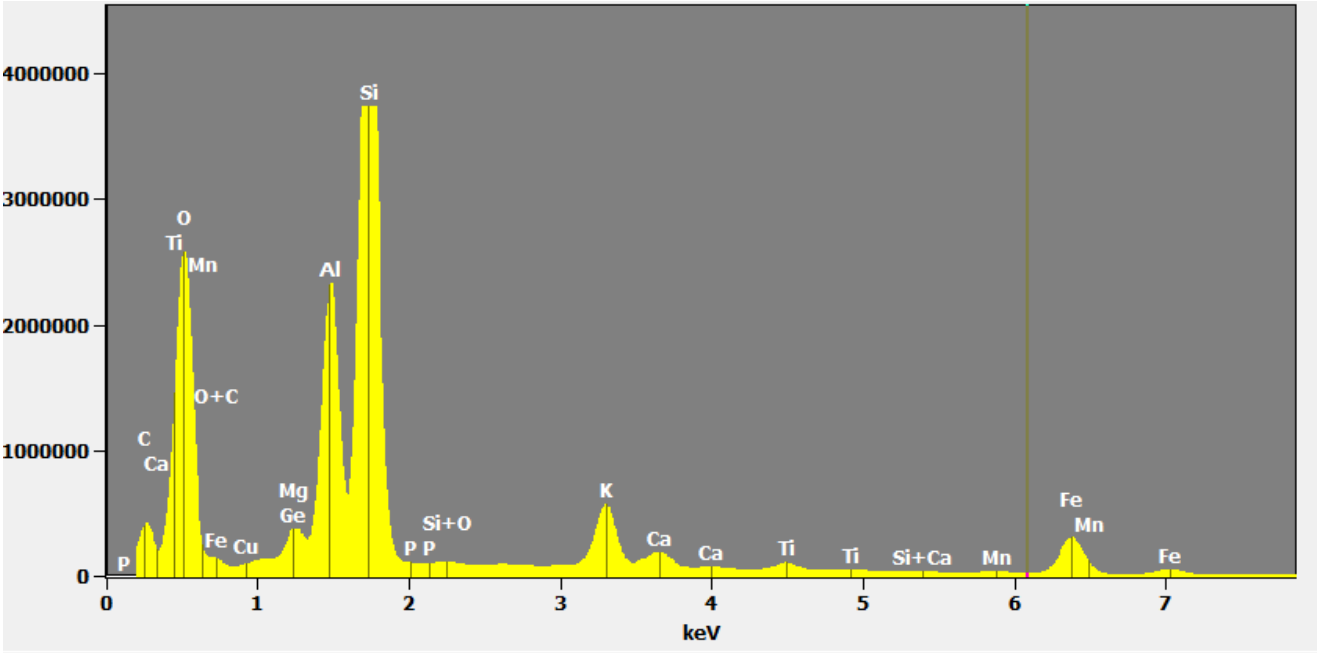


Figure 16 - SEM EDS spectrum analysis of the A horizon soil, collected from Chenqi in 2016. Elemental labels have been added to each of the peaks on the spectrum, to indicate the specific element at their associated absorbance levels.

An SEM EDS map of phosphorus of the A horizon is shown in Figure 17. The lighter-coloured areas of the map indicate the presence of phosphorus in the sample; there is one cluster of a phosphorus-containing compound in the sample, measuring approximately 40-50µm diameter. In addition to this larger cluster, there is some indication of phosphorus scattered in the background matrix of the soil, although given the scarcity of the light-coloured areas in the sample, it indicates that phosphorus is very limited within the A horizon sample from Chenqi. The phosphorus that has been visually identified in this sample has not shown up as brightly coloured, and therefore is understood to only be present in low concentrations.

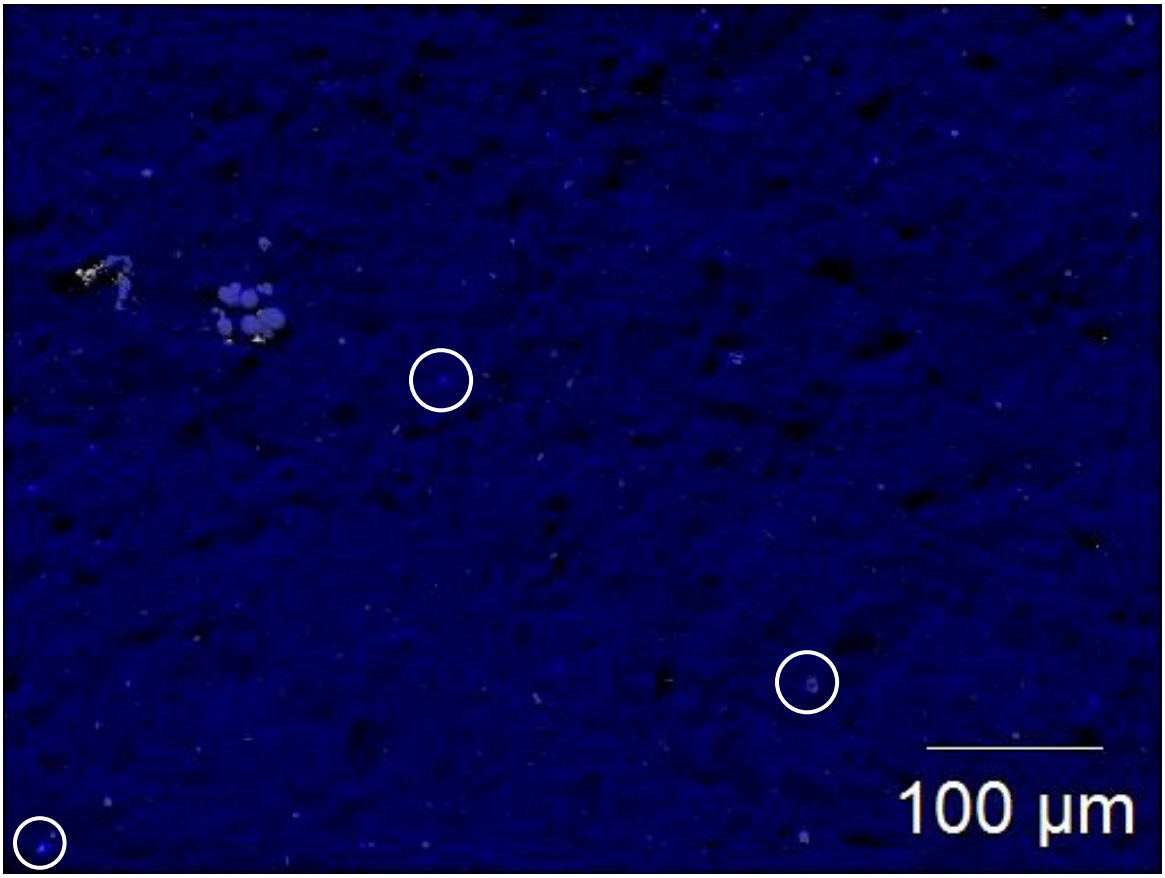


Figure 17 - SEM EDS map of phosphorus in the A horizon of the Chenqi soils collected in 2016. White circles have been used to highlight examples of phosphorus presence within the sample.

Figure 1Figure 18 includes two SEM EDS maps, which highlight the presence of calcium within the A horizon soil sample from Chenqi. Image A shows any calcium that is present within the sample, with the lighter green areas indicative higher concentrations of calcium. Examples of the areas of high calcium concentration have been highlighted in 18A using white circles; there appears to be a relatively high density of calcium species within the A horizon sample, When layered up with a phosphorus map to create image 18B, there are indications of the presence of some calcium-bound phosphorus species. These have been circled in white and are characterised in these SEM EDS maps as being areas of bright 'glowing' colour. In image 18B, there appears to be a very low concentration of calcium-bound phosphorus species in the A horizon sample; those species identified are very small and are distributed throughout the soil sample.

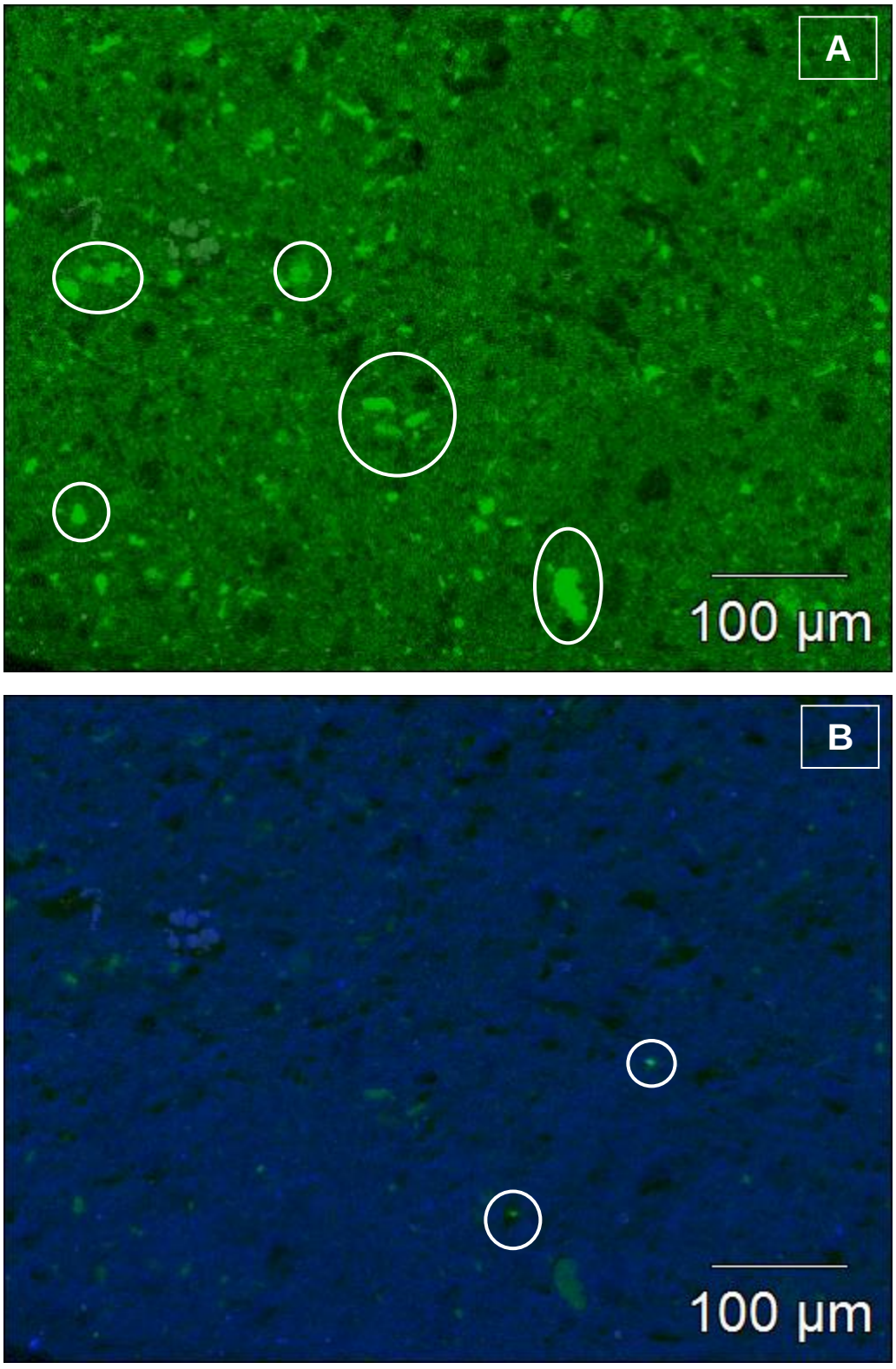


Figure 18 - Figure 18A is an SEM EDS image of calcium in the A horizon of the Chenqi soils, collected in 2016. White circles have been used to highlight examples of calcium species present within the sample. Figure 18B is a combined SEM EDS image, whereby 18A has been overlain with the phosphorus map from Figure 17; this allows for the identification of calcium-bound phosphorus species within the Chenqi A horizon sample.

Figure 19 encompasses two SEM EDS maps, both focusing on the presence of iron within the A horizon soil sample. Image A shows iron presence within the sample, with those areas of lighter red indicating a high iron concentration; there is a large cluster of iron to the left-hand side of the map, in addition to scattering of small, low-concentration iron particles throughout the soil matrix. There are some small particles with a higher concentration of iron, which have been outlined by white circles in image A of Figure 19. Layered SEM EDS elemental maps are used to identify compounds through the principles of colour-mixing; the blue coloured phosphorus map is layered on top of the red iron map to create image B in Figure 19. Any areas of magenta can be identified as iron-bound phosphorus, given the mixing of red (iron) with blue (phosphorus). The areas in image A identified as having high concentrations of iron are those that are bound to phosphorus; some of these have been circled in white in image B, further to the large cluster of iron-bound phosphorus located on the left-hand side of the image.

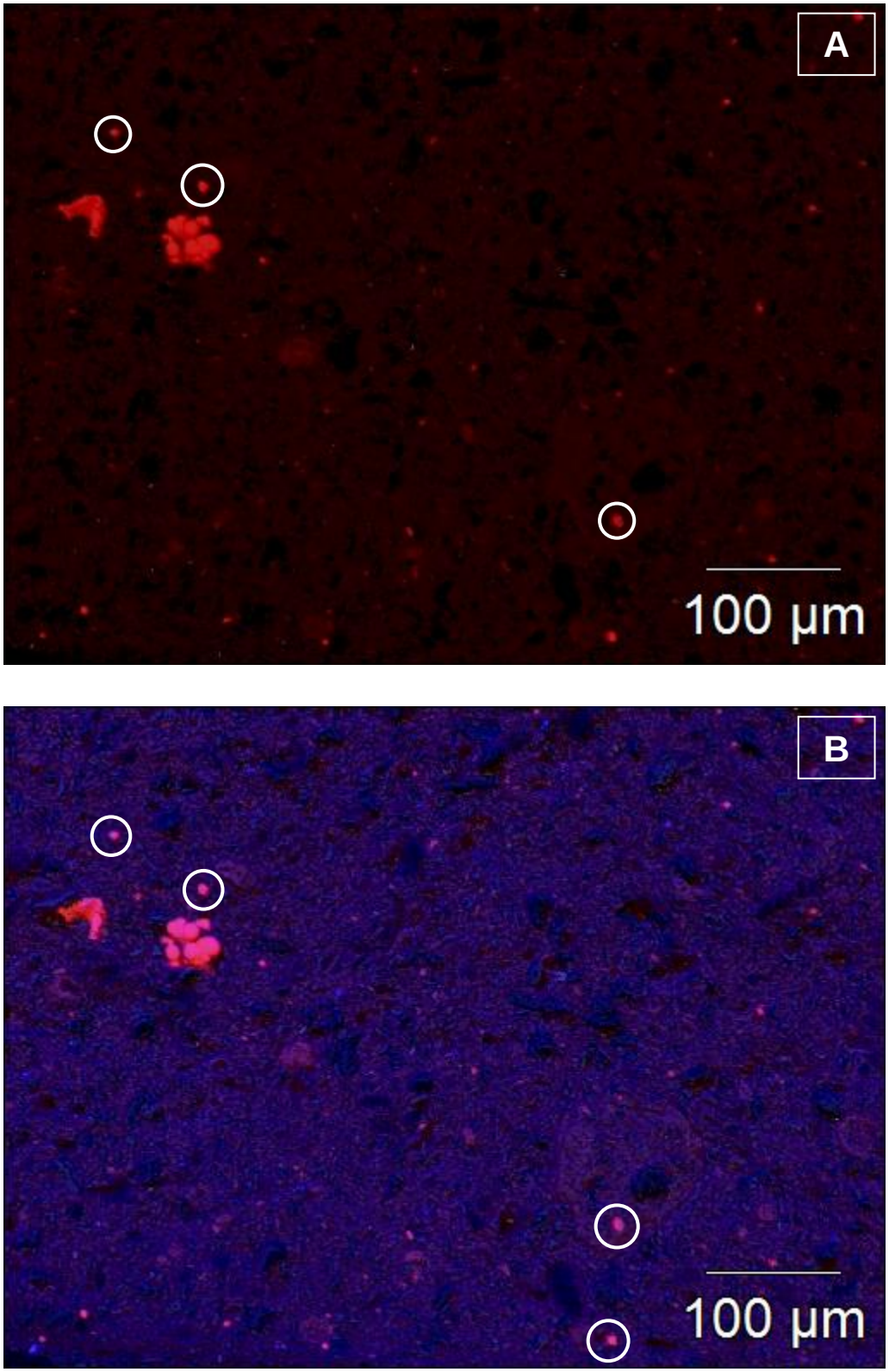


Figure 19 - Figure 19A is an SEM EDS map of iron in the A horizon of the Chenqi soils collected in 2016. White circles have been used to highlight examples of iron presence within the sample. Figure 19B is the SEM map of iron from Figure 19A with the map of phosphorus from Figure 17 overlain; this allows for the identification of iron-bound phosphorus species within the A horizon sample.

Figure 20 shows two SEM EDS maps, which examine the presence of aluminium within the A horizon. Image A indicates some aluminium particles in the sample, most of which are <50µm in size; these areas are circled in white in image A. There is additional scatter of aluminium particles through the background matrix of the soil, however given the darker green colour of these particles, it can be assumed they are a lower aluminium concentration than those particles circled in white. There is no aluminium present in the 50µm diameter cluster on the left-hand side of the image; the data from Figure 19 indicates that this cluster is purely iron-bound phosphorus. Image B in Figure 20 is a layered SEM EDS elemental map, where the phosphorus map from Figure 17 has been layered over the aluminium map shown in image A. Any turquoise coloured areas of image B are indicative of aluminium-bound phosphorus; there appears to be minimal aluminium-bound phosphorus in the A horizon sample.

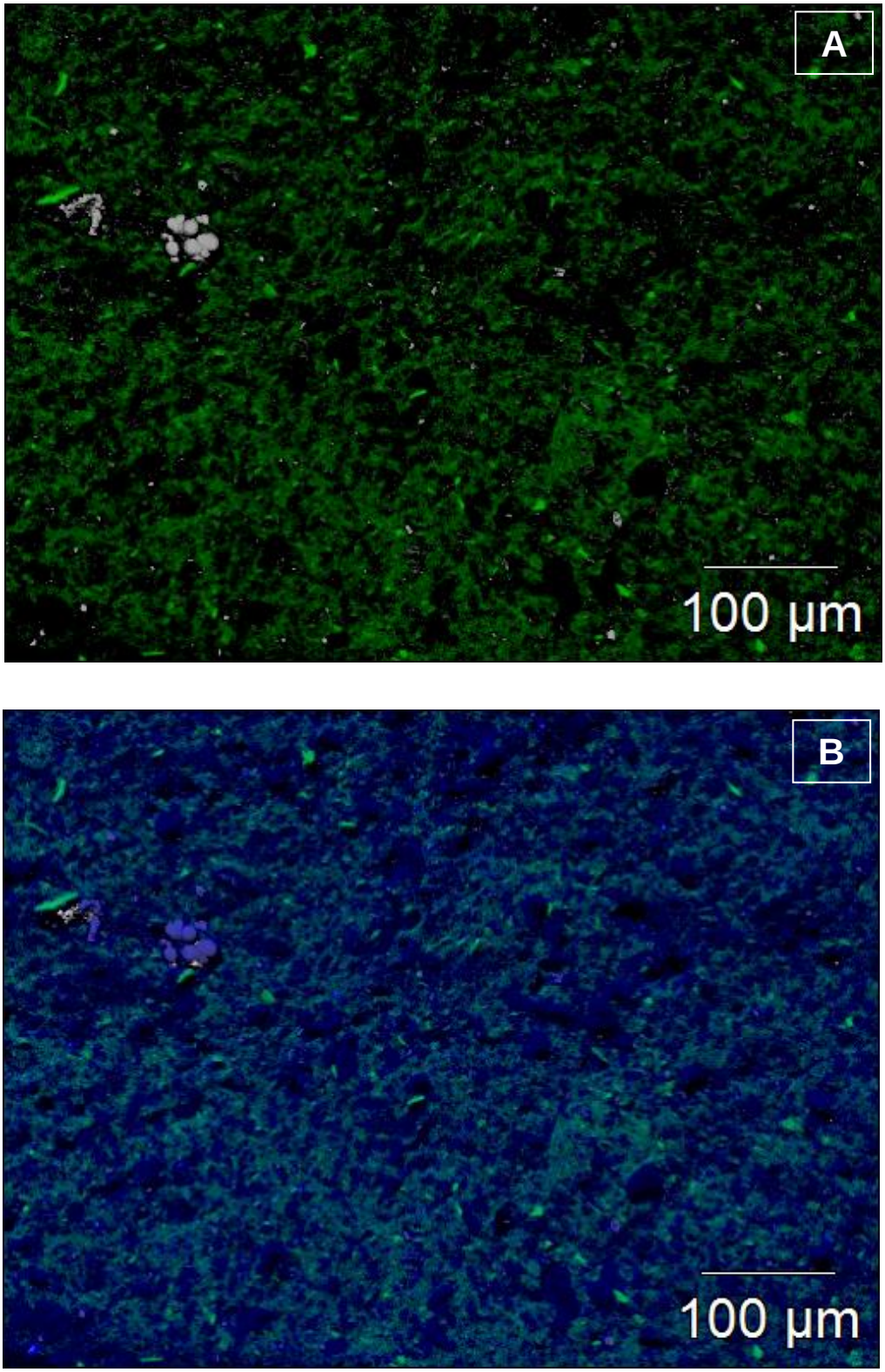


Figure 20 - Figure 20A is an SEM EDS map of aluminium in the A horizon of the Chenqi soils collected in 2016. White circles have been used to highlight examples of aluminium presence within the sample. Figure 20B is the SEM map from Figure 20A with the map of phosphorus from Figure 17 overlain; this allows for the identification of aluminium-bound species within the A horizon.

Figure 21 is a layered SEM EDS elemental map, which includes iron, aluminium and phosphorus maps. It seeks to identify any compounds that contain both iron and aluminium-bound phosphorus, as these phosphorus species have been identified as most abundant in Chenqi soils, as can be seen in Appendix B. In Figure 21, there appears to be no indication that any species which include both iron-bound and aluminium-bound phosphorus are present, as there are no areas that show blending of the colours used on the three respective elemental maps. As is present in Figure 19B, there is evidence of clusters of iron-bound phosphorus, in addition to the small areas of aluminium-bound phosphorus, as is seen in Figure 20B. However, these remain separate, and there are no obvious clusters that include both iron- and aluminium-bound phosphorus species.

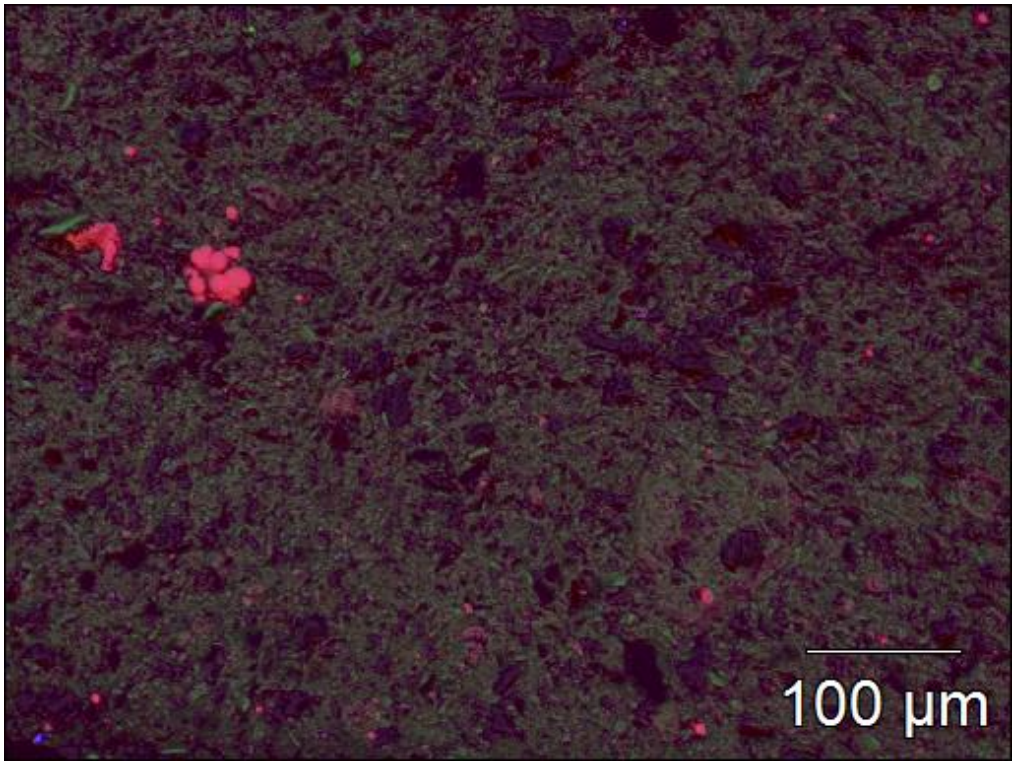


Figure 21 – A layered SEM EDS map of phosphorus (Figure 17), iron (Figure 18A) and aluminium (Figure 19A) in the A horizon of the Chenqi soils collected in 2016. The maps have been overlain to identify any compounds that contain both iron-bound and aluminium-bound phosphorus.

6.1.2. B HORIZON

A petrographic thin section of soil taken from the B horizon of the Chenqi samples was produced according to the method laid out in Section 5.3. On inspection using the SEM, it was found that the sample had not successfully impregnated with epoxy resin, due to the texture of the soil sample. Multiple attempts were made at producing a thin section, but none yielded a large enough surface of polished sample to be analysed using SEM EDS. Therefore, the decision was made to forgo analysis of the B horizon of the Chenqi soils, and instead focus on the samples collected from the A and C horizons.

6.1.3. C HORIZON

Figure 22 is a spectrum of SEM EDS data, which gives an understanding of the elemental composition of the C horizon of soil, collected from Chenqi in 2016. The spectrum data indicates that there is a very similar composition for both the A horizon and C horizon soils collected form Chenqi; silicon remains the dominant element in the C horizon, with high concentrations of aluminium, titanium, manganese and oxygen also present within the sample. In contrast to the A horizon sample, there is also a high concentration of chromium in the C horizon sample. In terms of phosphorus, the C horizon sample presents the same trend as the A horizon; there is almost negligible background concentrations of phosphorus in the C horizon.

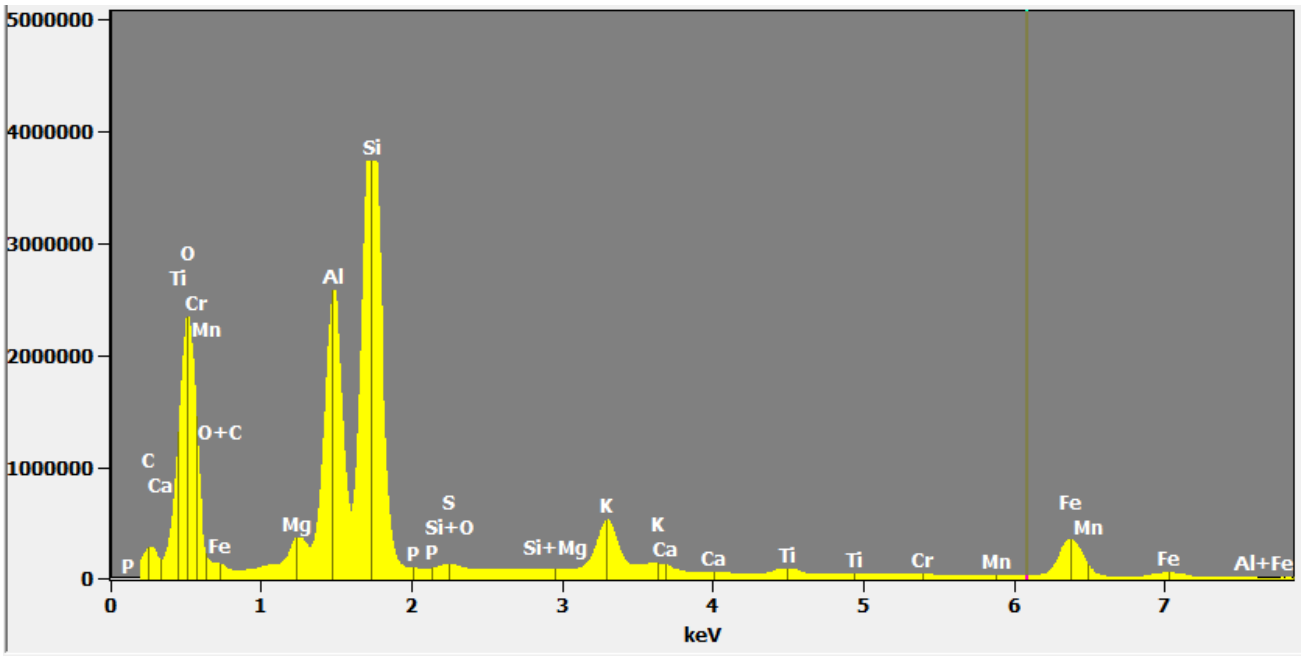


Figure 22 - SEM EDS spectrum analysis of the C horizon, collected from Chenqi in 2016. Elemental labels have been added to each of the peaks on the spectrum, to indicate the specific element at their associated absorbance levels.

The following results section displays the findings from the SEM EDS analysis on the C horizon soils from the Chenqi subcatchment. To provide a comparison with the A horizon, phosphorus, calcium, iron and aluminium are the focus of the analysis, however other elements were also selected for SEM EDS. The results of this additional analysis can be found in Appendix D.

The limitation of phosphorus in the C horizon sample becomes further apparent from the data presented in Figure 23. The SEM EDS map shows there to be only trace amounts of phosphorus present within the C horizon sample; these trace levels are circled in white in Figure 23. The C horizon of any soil is largely unaffected by surface applications of mineral fertilisers, given that the C horizon sits at around 50cm depth. The phosphorus identified using Figure 22 and Figure 23 is therefore likely to be naturally-occurring, and given the nature of the karst soils, it is highly-limited in its concentration. White circles have been used in Figure 23 to highlight any phosphorus particles; these particles are very small and appear to be randomly distributed in the soil sample. There are no larger clusters, which were present in the sample in the A horizon (Figure 17).

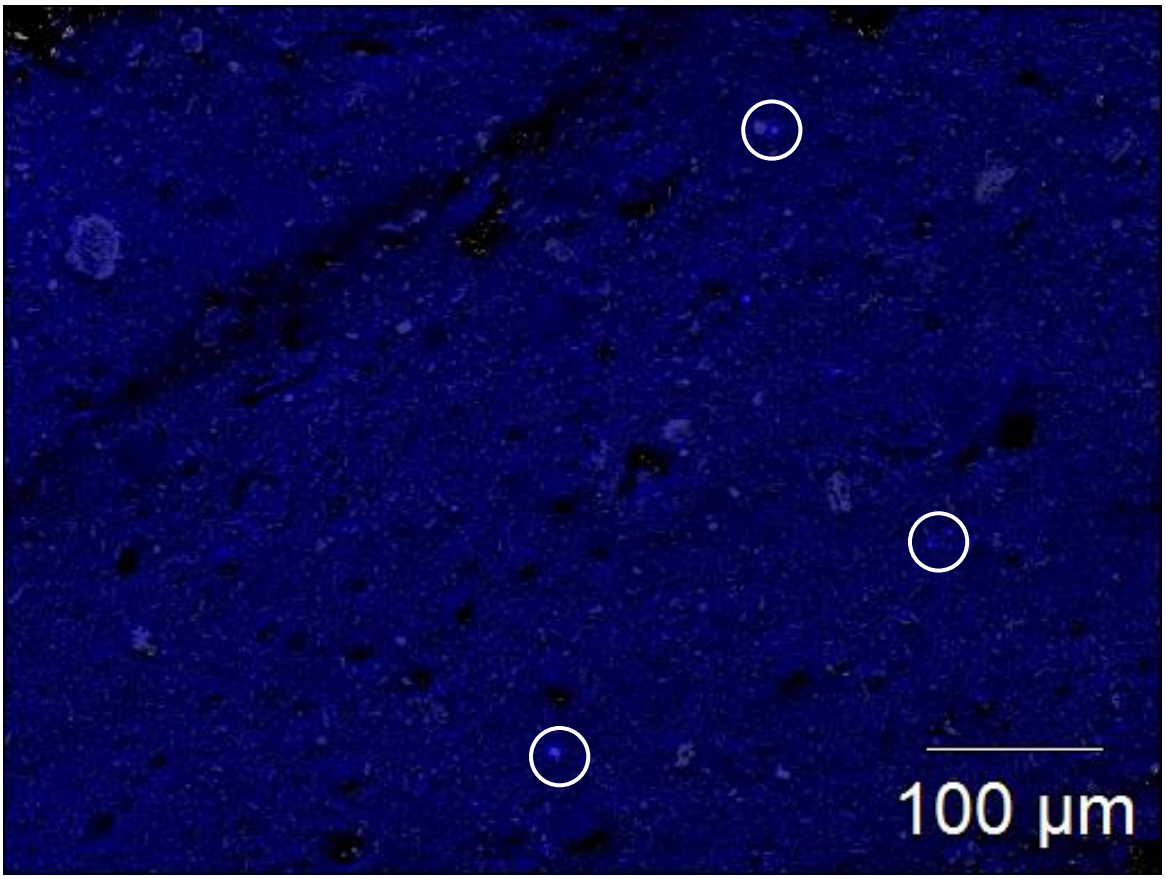


Figure 23 - SEM EDS map of phosphorus in the C horizon of the Chenqi soils collected in 2016. White circles have been used to highlight examples of phosphorus presence within the sample.

Calcium-bound phosphorus species were also analysed for in the C horizon soils from Chenqi. The elemental data presented in the spectrum in Figure 22 indicates that there is some calcium present within the sample, but it is at a lower concentration than Aluminium or Silica species. Figure 24 presents the SEM EDS analysis for the C horizon soil in relation to calcium species. Figure 24A shows calcium species in the C horizon, with the lighter and brighter areas indicating high concentrations of calcium. In the C horizon, there appears to be a very low density of calcium.

Figure 24A was overlain with the phosphorus map from Figure 23, to produce the map in Figure 24B. This image can be used to highlight calcium-bound phosphorus species in the C horizon of the Chenqi soil sample. There appears to be very low densities and concentrations of calcium-bound phosphorus within the C horizon sample; all calcium-bound phosphorus that has been identified in this sample is in the form of small particles (<5µm diameter) spread through the soil sample.

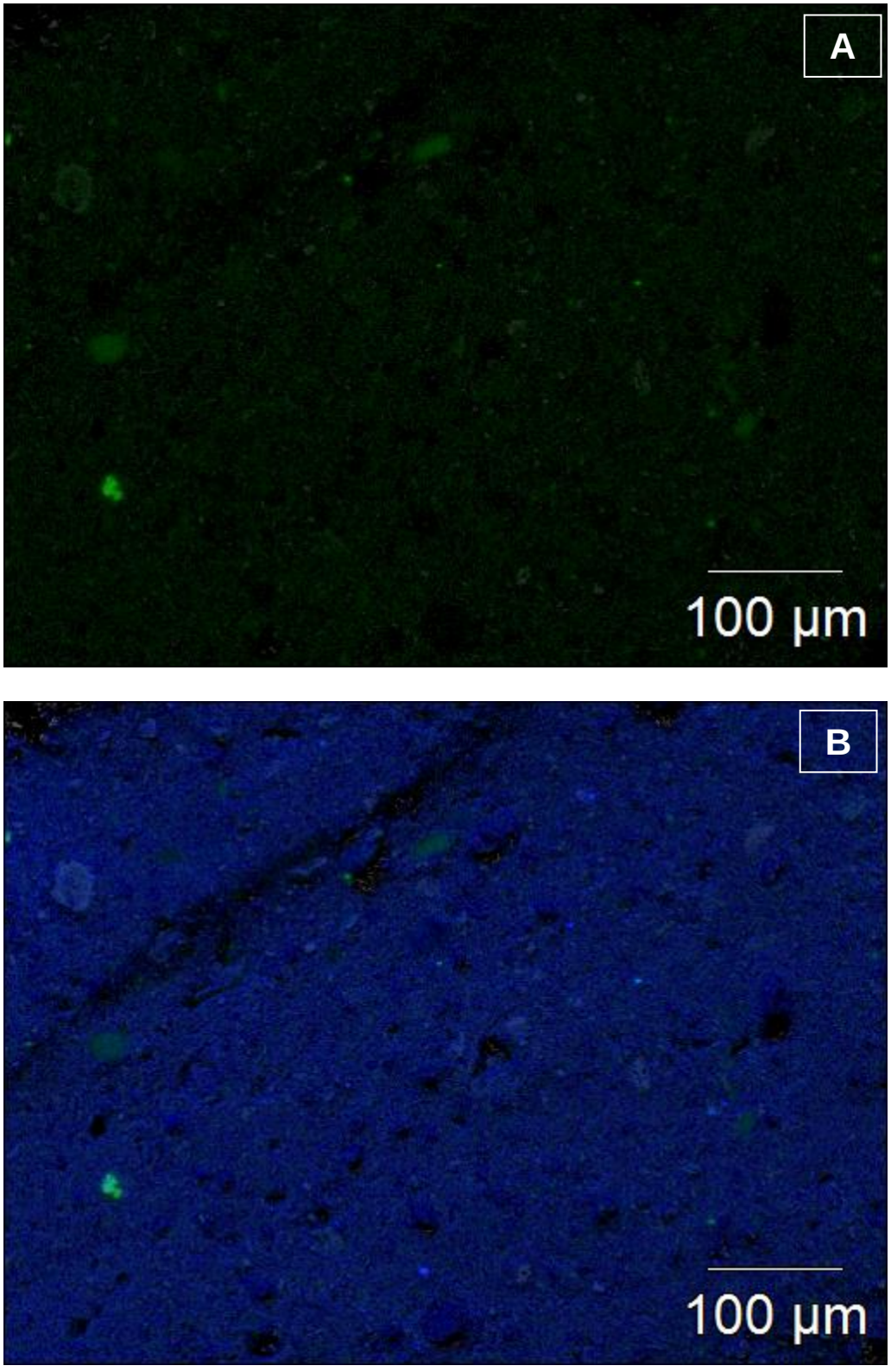
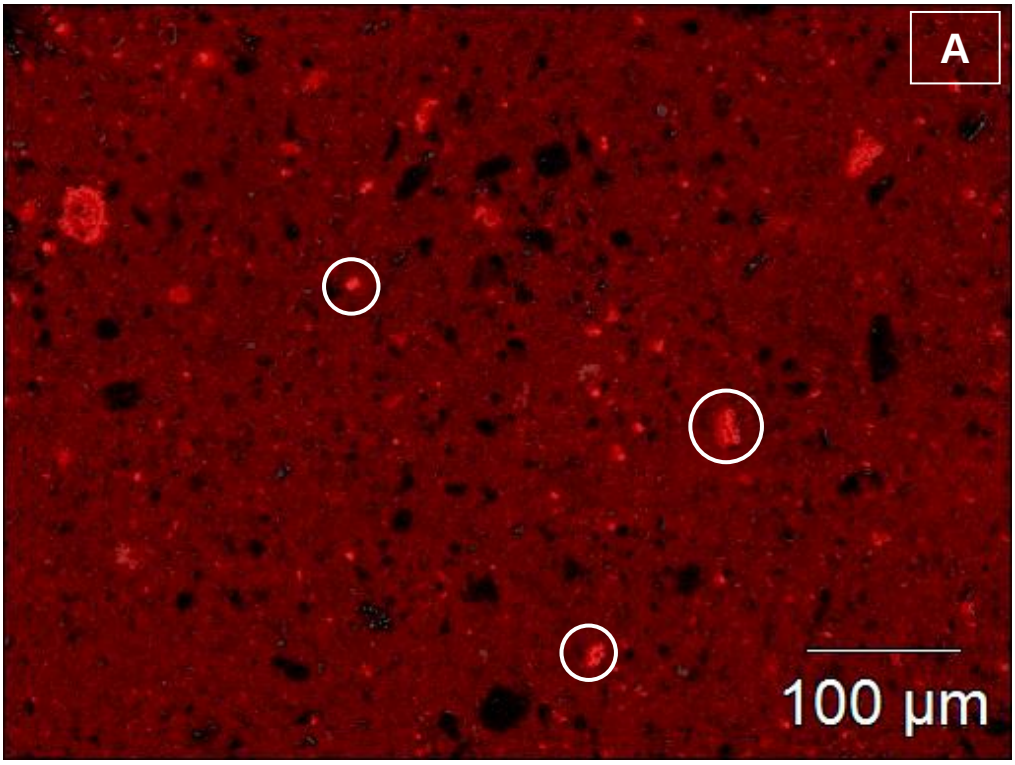


Figure 24 – Figure 24A is an SEM EDS map of calcium in the C horizon of the Chenqi soils collected in 2016. White circles are used to highlight examples of calcium presence within the soil sample. Figure 24B is the calcium map from Figure 24A, overlain within the SEM EDS map of phosphorus from Figure 23; this image allows for identification of calcium-bound phosphorus species within the C horizon.

The concentration of iron was also examined for the C horizon of the soils collected from Chenqi. The spectrum data in Figure 22 indicates that there is a relatively low concentration of iron present in the C horizon, but that this concentration is higher than that of phosphorus. Figure 25 is an SEM elemental map of iron in the C horizon sample; Figure 25A shows there are small particles of iron evenly distributed throughout the soil matrix. There are a range of different sized iron particles, ranging from approximately 30µm diameter, down to less than 5µm diameter.

The map from Figure 25A was overlain by the phosphorus map in Figure 23, to generate the map shown in Figure 25B. This map can be used to identify iron-bound phosphorus species that are present in the C horizon of the Chenqi soil sample. The layering of the blue phosphorus map and the red iron map result in any iron-bound phosphorus species being highlighted by a magenta colour on the SEM map. In Figure 25B, there are no iron-bound phosphorus species that can be visually identified. All the iron species identified in Figure 25A remain red coloured in Figure 25B, indicating they are simply iron particles, and are not bound to phosphorus in this soil sample.



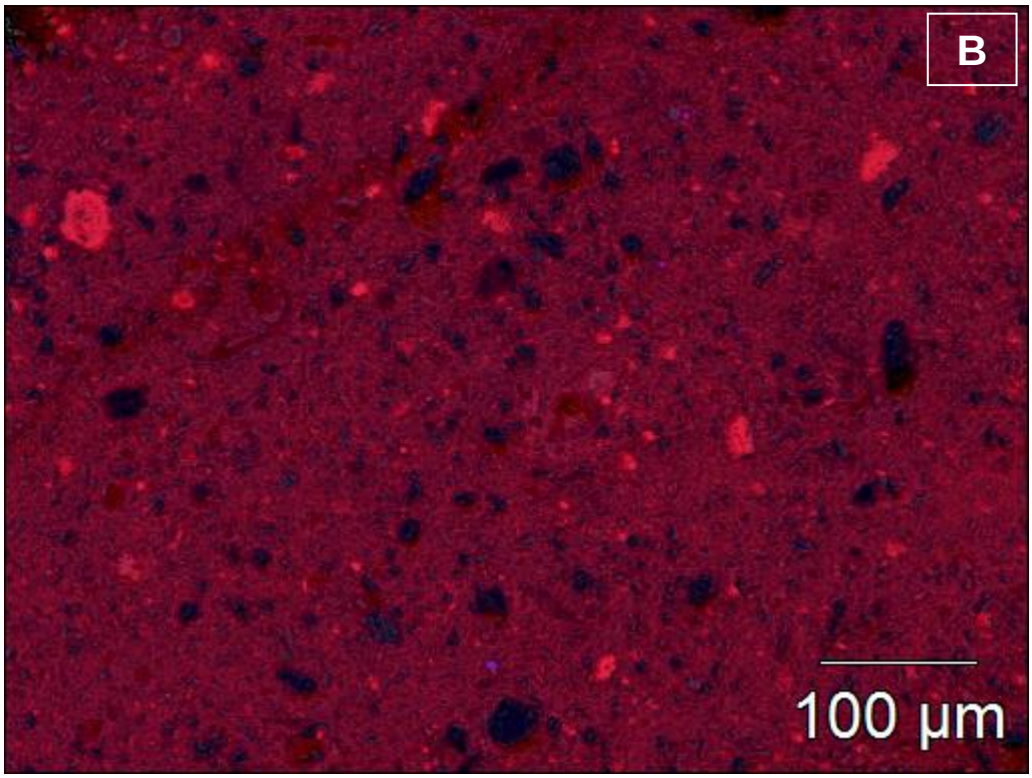


Figure 25 - Figure 25A is an SEM EDS map of iron in the C horizon of the Chenqi soils collected in 2016. White circles have been used to highlight examples of iron presence within the sample. Figure 25B is the map from Figure 25A, with the SEM EDS map of phosphorus from Figure 23 overlain; this allows for identification of iron-bound phosphorus within the sample obtained from the C horizon.

Aluminium was also examined for in the C horizon of the Chenqi soil samples, and the results are displayed in Figure 26. Figure 26A is the aluminium SEM EDS map for aluminium, where any particles of aluminium can be identified by a light green colour. There is evidence of aluminium particles within the C horizon, with some larger pieces having been circled in white in Figure 26A. Further to these larger particles, there are also <1µm particles of aluminium evenly distributed throughout the C horizon sample. This reinforces the conclusions drawn from the spectrum data (Figure 22), in that aluminium is one of the main elemental constituents of the C horizon.

The C horizon was also examined for the presence of aluminium-bound phosphorus, to better understand what species of phosphorus are held within the Chenqi soils. Figure 26B shows some indication of very small amounts of aluminium-bound phosphorus being present in the C horizon; these phosphorus species appear as a turquoise colour in Figure 26B and have been circled in white to aid identification. The particles of aluminium-bound phosphorus present in the sample are likely to be in very low concentrations, given the relatively dark colour they appear to be in Figure 26B. The layered SEM map also indicates that there is aluminium within the soil sample that is not bound to phosphorus; there is also evidence that phosphorus is present in the sample that is not bound to aluminium.

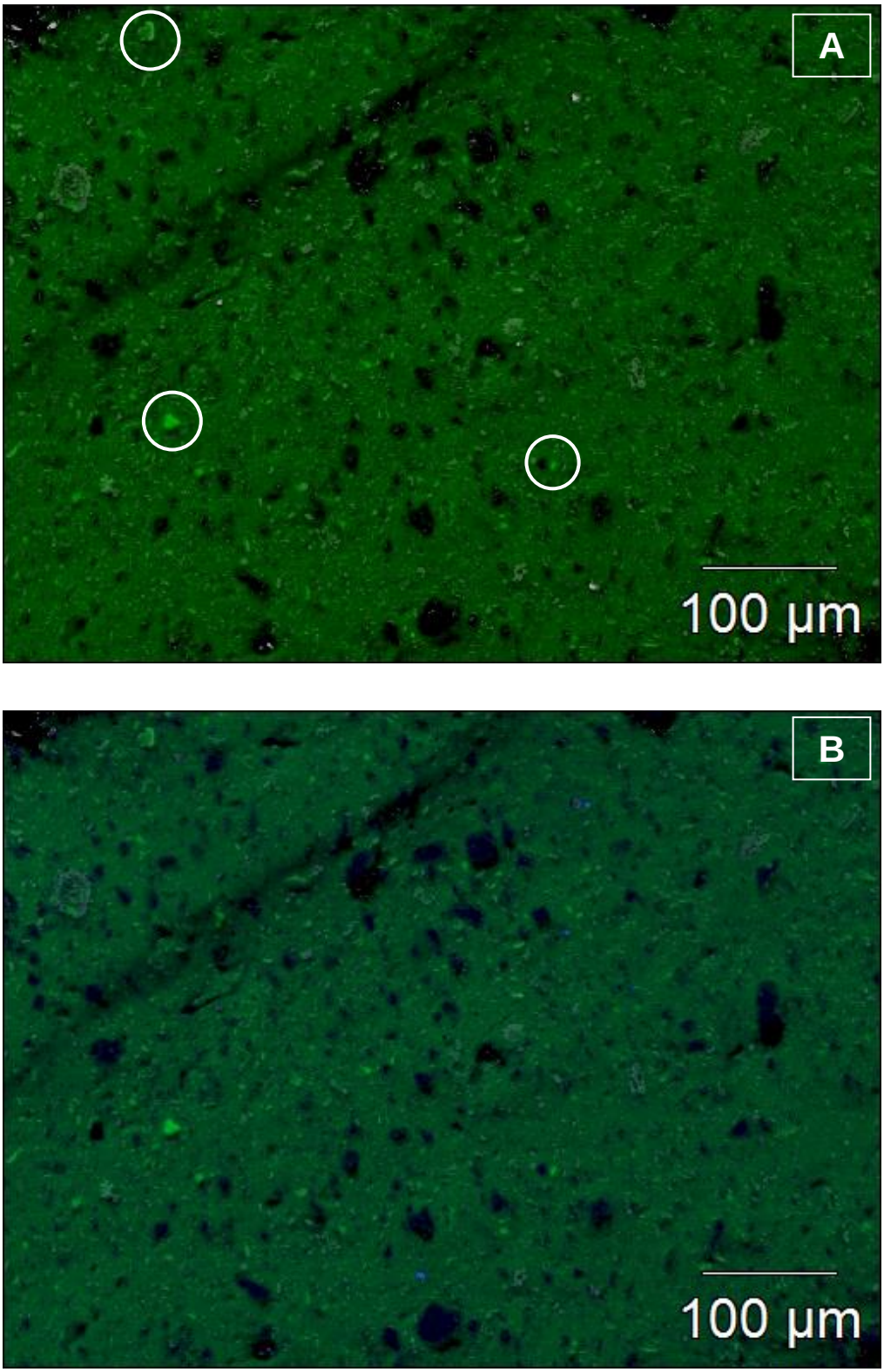


Figure 26 - Figure 26A is an SEM EDS elemental map of aluminium in the C horizon of the Chenqi soils collected in 2016. White circles have been used to highlight examples of aluminium presence in the C horizon. Figure 26B is the SEM EDS map of aluminium with the SEM EDS map of phosphorus from Figure 23 overlain, to allow for identification of aluminium-bound phosphorus in the sample taken from the C horizon.

Figure 27 is a multi-layered SEM map that seeks to identify any relationships between iron-bound and aluminium-bound phosphorus. The figure clearly shows that there is iron and aluminium within the C horizon, but very little of either element is bound to phosphorus. The iron particles appear as red in Figure 27, whilst aluminium is green and phosphorus is blue. Iron and aluminium appear to be prevalent within the soil, although the iron particles are much larger than the aluminium particles identified by the SEM analysis. There is an even distribution of both iron and aluminium in the sample, and there appear to be no areas where both aluminium and iron are present within the same particle or compound. Phosphorus is far more limited than iron and aluminium in the C horizon, with very few particles being identified by the SEM. There are two small pieces of phosphorus that can be identified in Figure 27, both of which have been highlighted in a white circle. The bright blue colour of these particles indicates that the phosphorus is neither iron, nor aluminium bound, as there is no mixing with the colours from those respective SEM maps. The phosphorus identified by this SEM analysis is either in the mineral form, or bound to another element, such as calcium; further analysis is required to confirm the nature of the phosphorus species in this soil horizon.

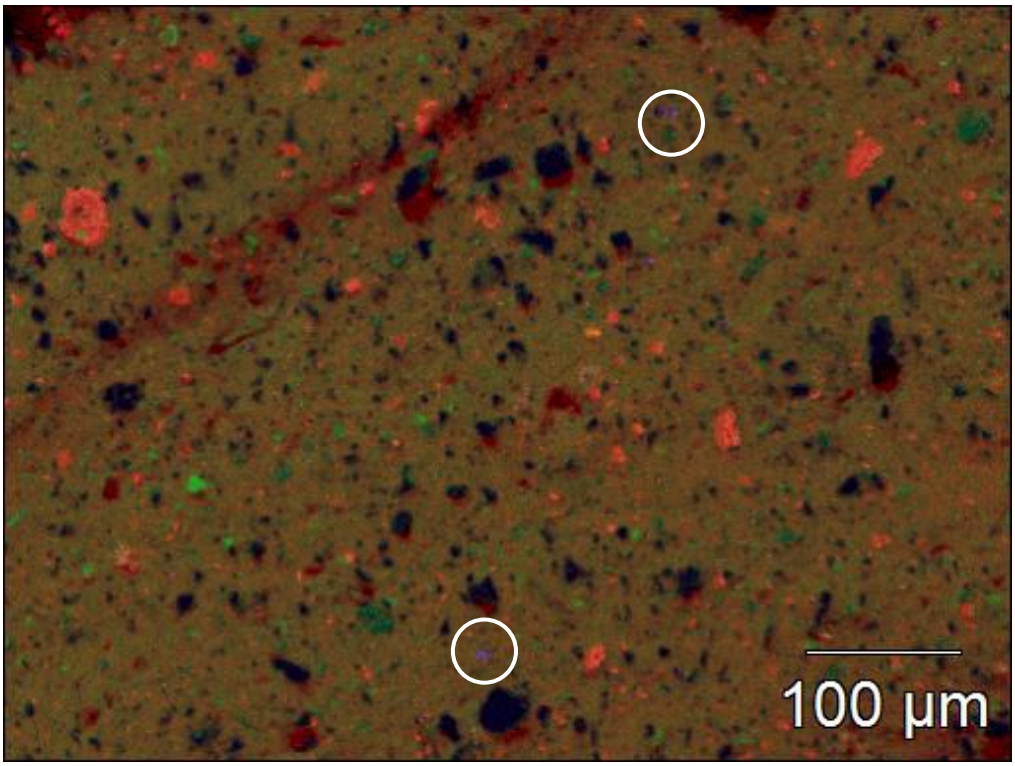


Figure 27 - SEM EDS map of phosphorus, iron and aluminium in the C horizon of the Chenqi soils. The phosphorus map, taken from Figure 23 has been overlain with the iron map from Figure 25A, and the aluminium elemental map from Figure 26A. The layering of different elemental maps allows for identification of any species containing phosphorus, iron and aluminium. White circles have been used to highlight the phosphorus particles identified in this sample.

6.2. PLANT HEALTH AND GROWTH QUALITY

Throughout the process of growing the *Erigeron acris* for analysis of the uptake of phosphorus, the plant health and overall growth quality was monitored and recorded. Phosphorus deficiency in plants is often characterised by purple-brown mottling or discoloration to the leaves, as can be seen in Figure 29.

The control plants, grown in potting compost throughout the experiment, can be used for comparison with the plants grown in the Chenqi soils, to assess the overall plant health and quality of growth. Figure 28 shows photos of the control plants, taken at the end of the experimental process. The control plants remained healthy throughout the experiments, with dark green leaves and consistent sprouting of new shoots and leaves. There were no visible indications of phosphorus limitation in the control plants, such as purple-brown mottling on the leaves, or a purple discolouration of the stems. This indicates that, at no point during the experiments, were the control plants limited with respect to phosphorus.

Figure 28 - Erigeron acris grown in potting compost, as a control sample for the experiment. These images are taken after 12 weeks of growth, however all plants were monitored throughout the experimental process for visible signs of phosphorus deficiency, such as purple coloured stems and purple-brown mottling on the leaves.

The Erigeron began to show some signs of phosphorus deficiency after being planted in the Chenqi soils for 3 weeks; this was mainly manifesting itself as faint purple mottling appeared at the base of the stems. This was present across the A, B and C horizon soils, for the 20µM and 40µM Oxalic acid treatments, in addition to the untreated Chenqi soil. There was no clear difference in the mottling between the different soil horizons or acid treatments, but all experimental treatments showed signs of phosphorus deficiency.

After the full growth term of 12 weeks, prior to the plant biomass being digested and processed for further analysis, the plants were fully examined for phosphorus deficiency. As was found in the earlier stages of growth, phosphorus deficiency was identified in all the Chenqi soil horizons under the 20µm and 40µm oxalic acid treatments, in addition to the soils which received no acid treatment. The *Erigeron acris* grown in Chenqi soils that were untreated with acid displayed signs of phosphorus deficiency on all the plant stems, in addition several leaves having purple tips or undersides. Evidence of this can be seen in Figure 29, which capture phosphorus deficiency in plants grown in Chenqi soils with no additional application of oxalic acid.

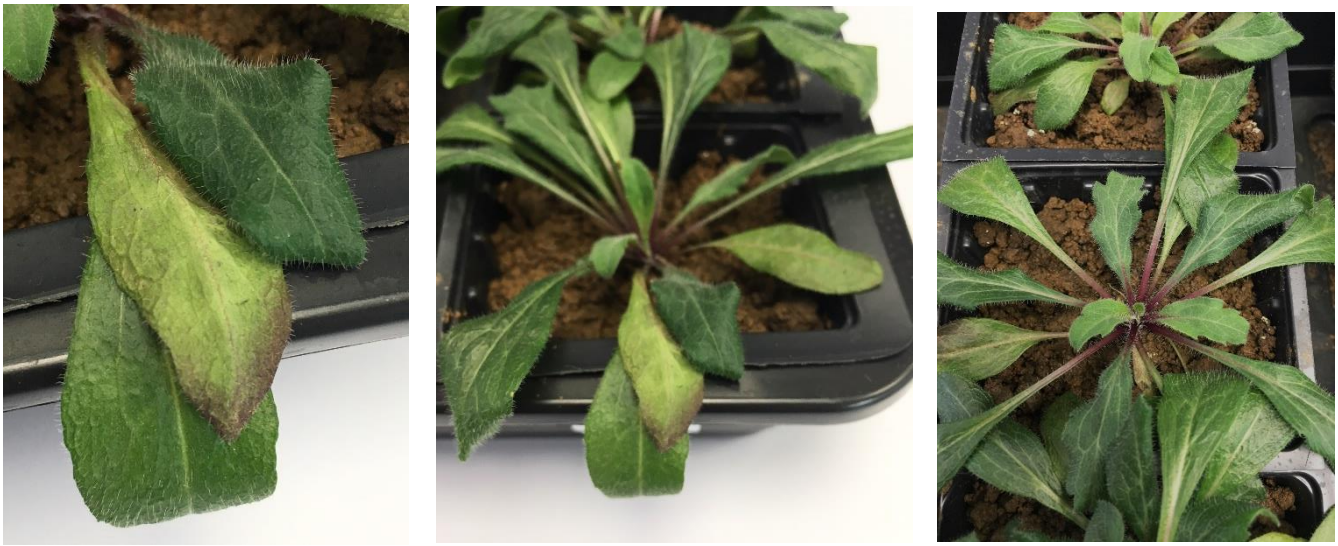


Figure 29 - Erigeron acris grown in Chenqi soils with no additional treatments of oxalic acid applied. Signs of phosphorus deficiency are visible and take the form of purple-brown mottling on the leaves, in addition to a dark purple colour appearing on the stems and shoots.

The *Erigeron acris* grown in Chenqi soils treated with an application of 20µm oxalic acid also showed some indications of phosphorus deficiency. Plants grown with 20µm oxalic acid showed visual signs of being healthier and less affected by phosphorus limitation in the soil. The plant leaves were darker green than those plants grown with no oxalic acid treatment, with the plants treated with 20µm oxalic acid bearing a closer resemblance to the control plants, which grew very well and showed no indication of phosphorus limitation. Although the overall plant health and growth quality of the plants grown using 20µm oxalic acid was better, there remained some indications of phosphorus limitation. Although a dark green colour, the leaves still showed some indication of phosphorus limitation, with dark purple-brown mottling across the top surface and underside of several leaves on the plants. This mottling can be seen in the central image of Figure 30, with the purple discolouration of the stems visible in the right-hand photo. The same discolouration and indications of phosphorus limitation could be seen across plants grown in all three soil horizons treated with 20µm oxalic acid.

Figure 30 - Erigeron acris grown on Chenqi soils treated with 20µM oxalic acid. The plants show obvious signs of phosphorus deficiency, including purple mottling to the leaves, and discoloration of the stems and shoots.

40µm oxalic acid was used as a treatment for Chenqi soils, across all three soil horizons. The *Erigeron acris* grown in these soils presented similar overall plant health and growth quality to those to those plants grown in soils treated with 20µm oxalic acid. The leaves of the plants took on a dark green colour, the same as those grown in the control experiments and in the soils treated with 20µm oxalic acid. As can be seen in Figure 31, there remained some evidence of the phosphorus limitation, in that a very small number of leaves on the plants had purple-brown mottling and discolouration on the surface and underside of the leaves. Most of the stems and shoots of the plants took on a purple colour, once again indicating the depletion of phosphorus in the growth medium. There were no signs of phosphorus limitation on the plants grown in the potting compost as control samples; this indicates that the purple mottling is related to the low natural concentrations of phosphorus found in the Chenqi soils, as opposed to a natural colouring of the plant that develops over time. There was a visible difference in plant health between the soils treated with 40µm oxalic acid and the Chenqi soil that were not treated with oxalic acid, however there was no noticeable difference in the health or growth of the plants treated with 20µm oxalic acid.

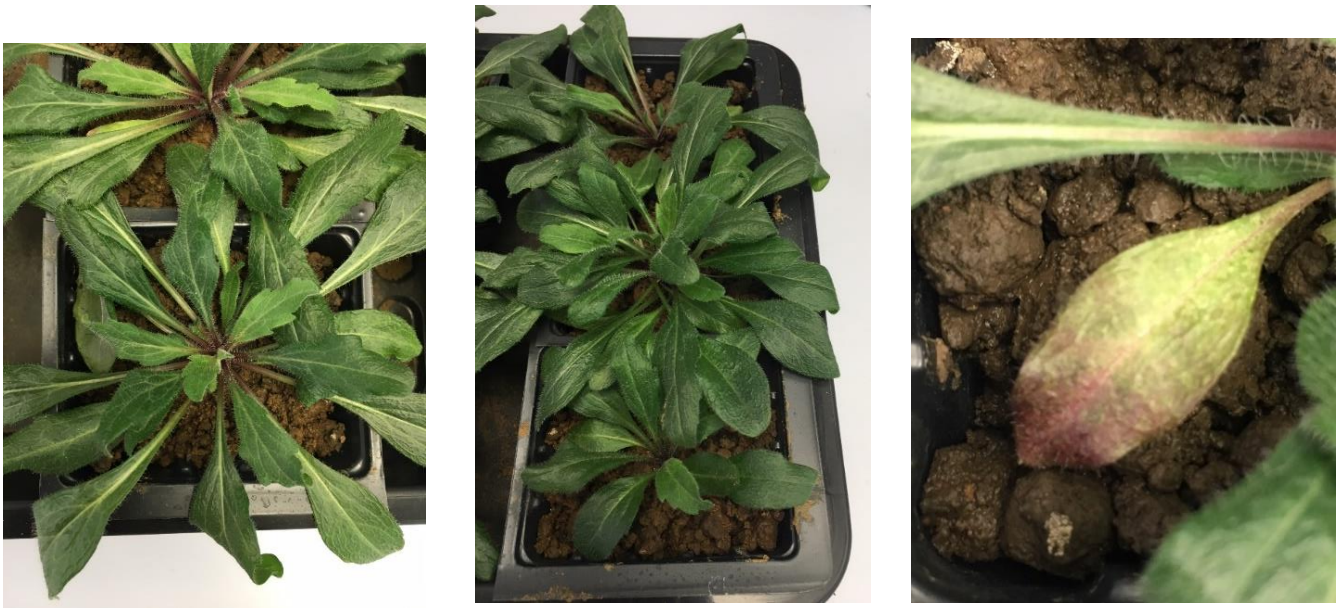


Figure 31 - Erigeron acris grown on Chenqi soils with an additional 40µm oxalic acid treatment applied. The plants show signs of phosphorus limitation and deficiency, such as purple-brown mottling of the leaves, in addition to the stems taking on a dark purple colour.

6.3. PLANT BIOMASS CHANGE

The data was collected for all soil horizons and treatments, for each of the repeat seedlings planted; this data is presented in Figure 32, and shows the mean average biomass change for each soil treatment. Range bars indicate the maximum and minimum values of biomass change recorded for seedlings grown in each of the soil treatments

The results presented in Figure 32 indicate there is no obvious relationship between the concentration of oxalic acid applied to soils and the total biomass change observed. The control sample (C) showed a negligible mean change in total plant biomass, although there is a negative range bar that indicates at least one of the seedlings lost biomass during the growth period. There was no limitation of phosphorus in the control sample soils, and therefore there is no clear explanation for the negative change in biomass. There is a wide spread of data for the Chenqi soils, ranging from the highest mean plant biomass change of 101% for plants grown in the 0A soils, to -40% for plants grown in the 40C soils. In addition to the overall spread of data between each of the soil treatments, there is also a large range of values generated for most of the soil treatments. Soil treatments 0A, 0C, 20B, 20C and 40A show the biggest range between the maximum and minimum values of plant biomass change. These treatments have ranges in data values of >100%, resulting in overlap in the results for many of the samples.

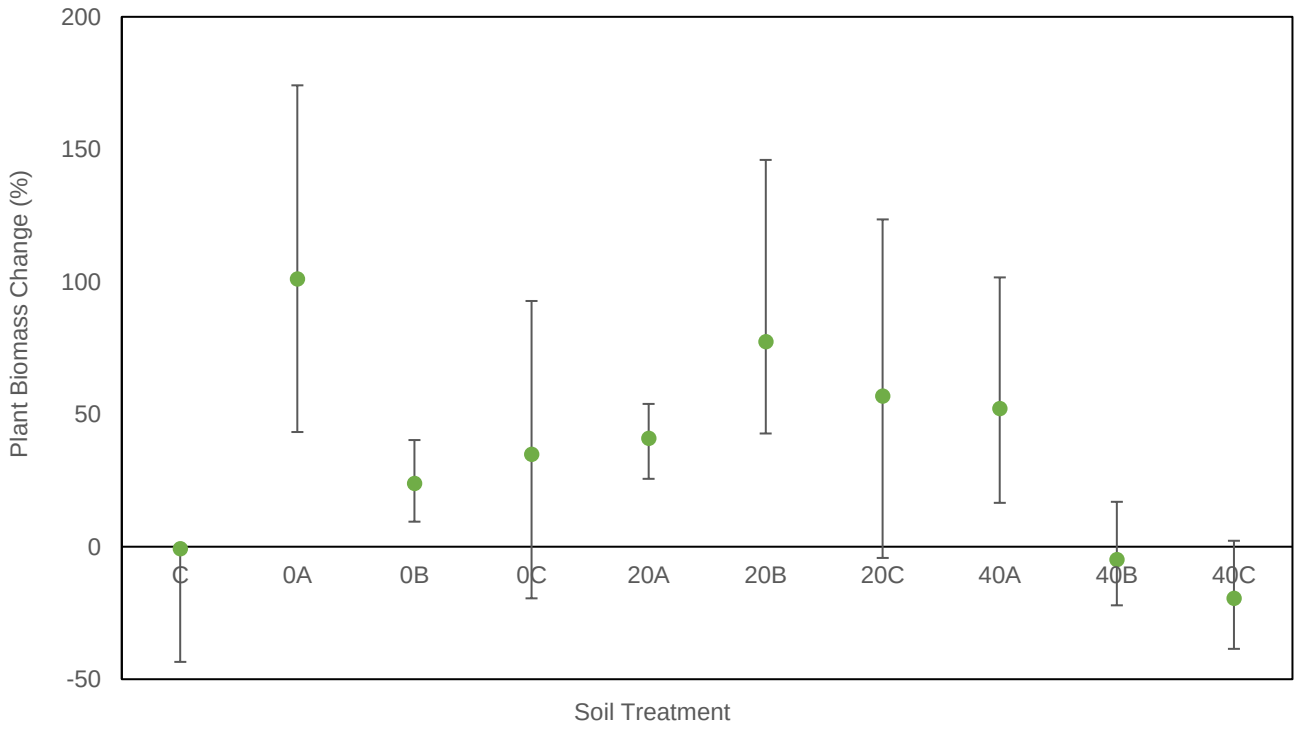


Figure 32 - Plant biomass change for seedlings in Chenqi soils, calculated as: (Final Plant Mass - Initial Plant Mass)/Initial Plant Mass x 100. The green markers indicate the mean average biomass change for plants grown in each soil treatment. Range bars indicate the total range of values for each of the soil treatments.

There is no clear trend between the concentration of oxalic acid applied to Chenqi soils and the total plant biomass change, in that there is no increase in plant biomass with increasing concentrations of oxalic acid, as was originally hypothesised. 0A shows the highest mean plant biomass change, followed by 20B and 20C. Those soil treatments with the lowest mean plant biomass change are 40C and 40B, both of which saw a negative change in plant biomass; these soil treatments also had a much smaller range in values. Further to exploring the influence of oxalic acid concentration on the plant biomass change, there is also the opportunity to examine how different soil horizons respond to the application of oxalic acid. From the data in Figure 32 it is apparent that there is no trend in the horizon to which acid is applied, and the mean plant biomass change. In soils where no oxalic acid was applied (0A, 0B, 0C), the A horizon soil had the highest plant biomass change, and the B horizon the lowest change. In soil samples 20A, 20B and 20C, which were treated with 20mM oxalic acid, it was found that 20B seedlings had the greatest increase in plant biomass, although the range bars overlap with those for 20A and 20C seedlings. For soils treated with 40mM oxalic acid, plants grown in the A horizon saw the greatest increase in plant biomass, whilst those in the C horizon on average lost biomass during the growth period. For 40B and 40C, the range bars are predominately overlapping, with both soil treatments showing that mass loss had occurred for some of the seedlings cultivated. The data presented in Figure 32 show a large amount of overlap between different soil treatments, and therefore statistical analysis techniques are required to better examine the results, to determine if any trends are statistically significant.

Table 6 - t-Test results for plant biomass change. The t-Test seeks to identify if the mean values of two variables/samples are significantly different. A two-tailed t-Test means results must satisfy one of the following criteria: the t Stat < -t Critical two-tail or t Stat > t Critical two-tail. If either test is satisfied, then the null hypothesis ($\mu_1 - \mu_2 = 0$) is rejected, and it can be concluded that the variable means differ significantly.

Variable 1	Variable 2	t Stat	t Critical two-tail	t Stat < - t Critical two-tail	t Stat > t Critical two-tail
0A	20A	1.526889	4.30265273	No	No
0A	40A	1.057727	2.776445105	No	No
20A	40A	-0.4205	2.776445105	No	No
0B	20B	-1.51112	2.776445105	No	No
0B	40B	1.966577	2.776445105	No	No
20B	40B	2.273063	2.776445105	No	No
0C	20C	-0.44895	2.776445105	No	No
0C	40C	1.572535	2.776445105	No	No
20C	40C	1.967578	2.776445105	No	No

Two-tailed t-tests were used as the primary form of statistical analysis, to identify if the difference between the mean averages of two different variables were statistically significant. The t-test is prefaced by an f-test, which identifies whether the variance in the spread of data is equal or unequal, and subsequently the appropriate t-test that should be used for analysing the data set; the results of the F-tests can be found in Appendix E. The results of the t-tests are displayed in Table 6, and as a two-tail t-test has been used, there are two conditions that could be fulfilled to show statistical significance between two variable means. The influence of oxalic acid concentration on plant biomass was analysed using the t-tests, and conclusions drawn based on whether one or both conditions were met. In all combinations of soil treatments tested using the t-test, there was no statistically significant difference found between the mean of the two variables. This shows that although some soil treatments saw a higher mean biomass change than others, it is not possible to say conclusively that there is a relationship between the concentration of oxalic acid and the increase in plant biomass observed.

6.4. TOTAL PHOSPHORUS CONCENTRATION IN PLANT BIOMASS

Figure 33 displays the results of the *Gallery Plus* analysis on the plant biomass, where total phosphorus concentration was measured in the plant leaves and roots of seedlings grown in each of the experimental soil treatments. The samples were run on the *Gallery Plus* using both the "Phosphorus High" and "Phosphorus Low" tests, but the "Phosphorus Low" test was found to provide a better coverage of data given the low concentrations of total phosphorus found in the plant biomass. A small number of the samples could not be detected using the "Phosphorus Low" test, and therefore results from the "Phosphorus High" test were used to fill in gaps in the final data.

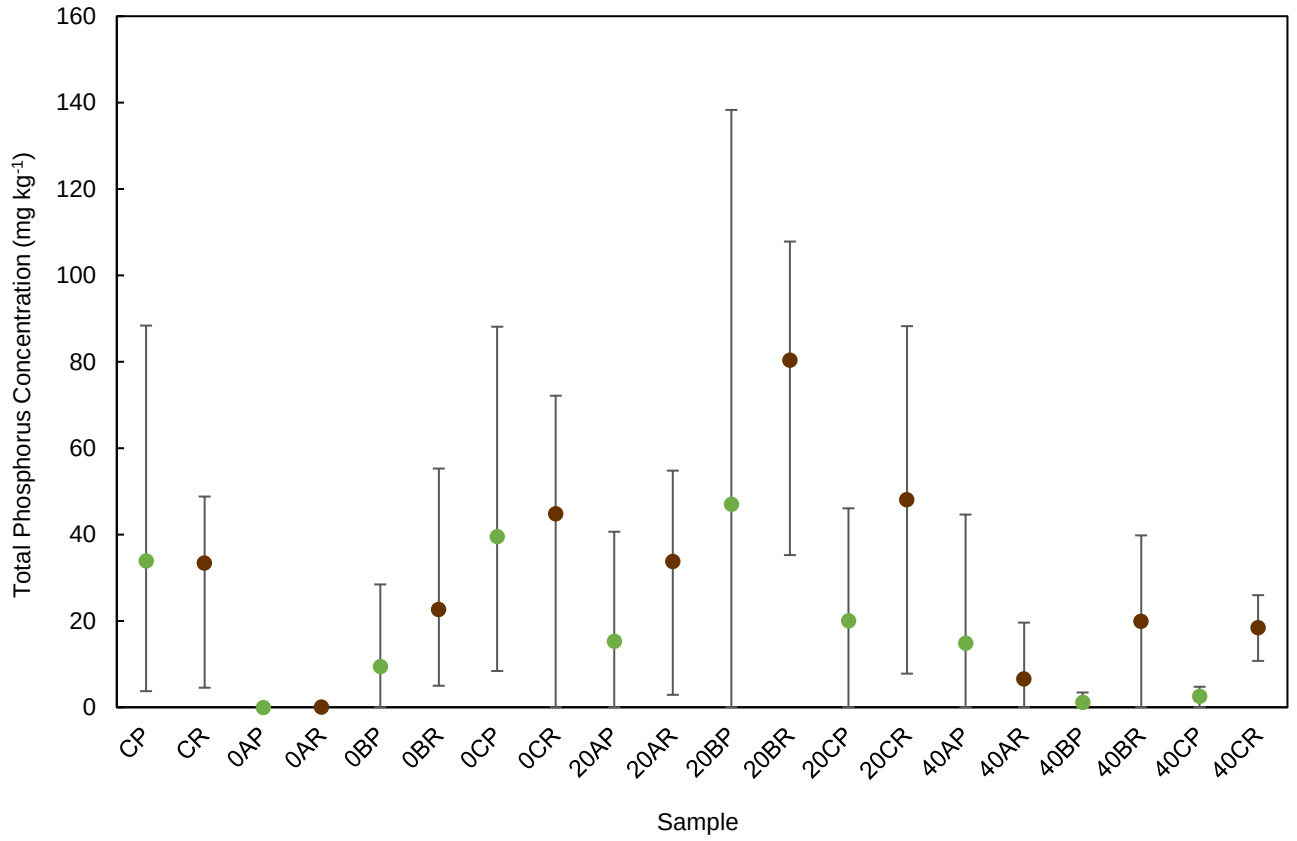


Figure 33 - Total phosphorus concentration in plant biomass, measured in mg kg^{-1}. Green markers indicate samples of above-surface biomass (leaves and shoots), whilst brown markers indicate samples of subsurface biomass (roots). The mean average total phosphorus concentration has been plotted, with range bars to indicate the minimum and maximum values measured for each sample.

There are a number of trends and patterns in the data presented in Figure 33. Samples CP and CR are seedlings grown in the control soil, an autoclaved potting compost. The mean average phosphorus concentration for the leaves and shoots is 33.9 mg kg^{-1} whilst for the roots it is 33.3 mg kg^{-1} (3.s.f.); the range for the control root samples is far smaller than the range for the control leaves and shoots, which

has a maximum concentration of 88.3 mg kg^{-1}. Excluding the seedlings grown in 40A soil, there is a general trend observed that the total phosphorus concentration of the root material is higher than the corresponding leaf and shoot biomass. In some soil treatments, such as 20B and 20C, this difference is more profound, whilst in others it is only a small variation in phosphorus concentration, such as the 0C soils. In terms of overall range in the dataset, the seedlings grown in 0A soils contained 0 mg kg^{-1} of phosphorus, which was the lowest concentration recorded. It is unclear whether the sample contains no phosphorus, or if the concentration is negligible or too low to be picked up in analysis. The highest concentration of phosphorus was found in the 20B soils, in the root matter, where the mean concentration measured 80.3 mg kg^{-1}.

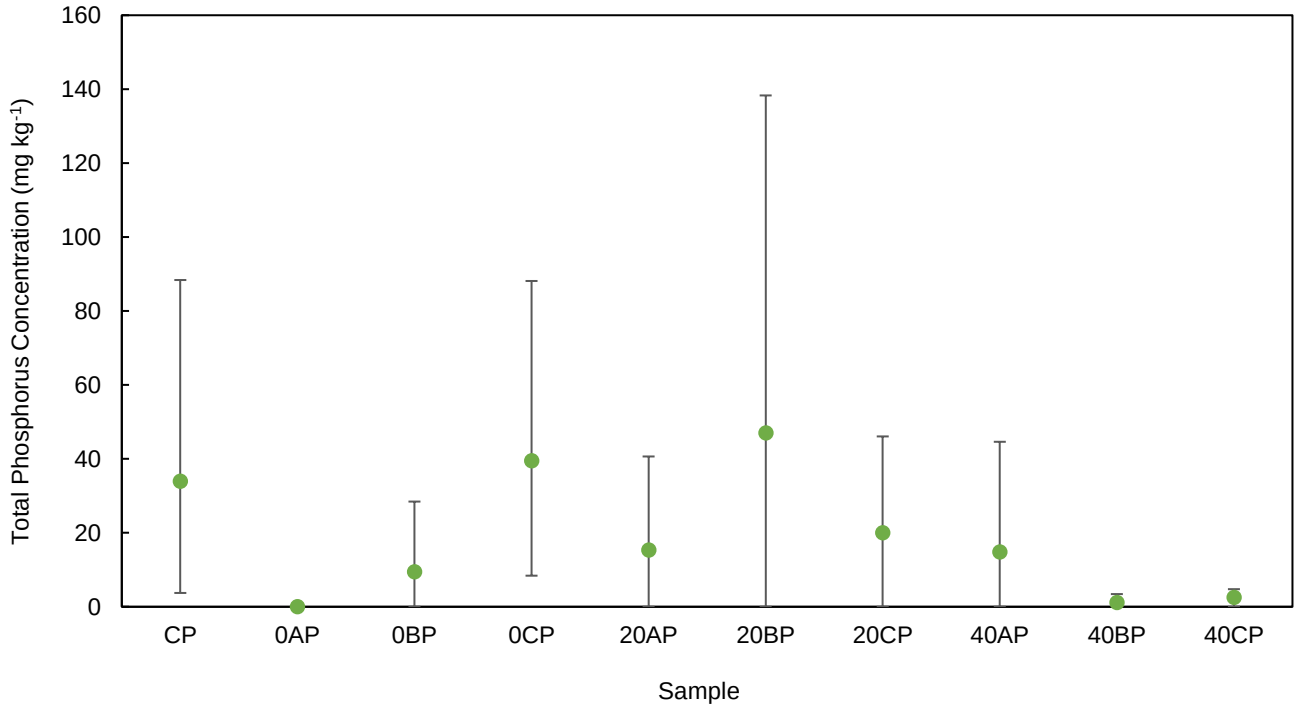


Figure 34 - Total phosphorus concentration in leaves and shoots of the samples, measured in mg kg^{-1}. The mean average total phosphorus concentration is indicated by the markers, with range bars used to illustrate the maximum and minimum values.

Figure 34 shows the results of total phosphorus analysis for the leaf and shoot samples grown on each of the soil treatments, whilst Figure 35**Error! Reference source not found.** displays the total phosphorus concentration of the roots of the seedlings. The seedlings grown on B horizon soils with 20mM oxalic acid have the highest concentration of phosphorus found in the leaf and shoot matter, however the 20B soils also present the largest range; given this large range in data, it is difficult to draw any firm conclusions about trends in the data. Similarly, seedlings grown in the B horizon soil treated with 20mM oxalic acid also have the highest concentration of phosphorus in the root matter, as is shown

in Figure 35**Error! Reference source not found.**. The root matter data shows a smaller range in values than the leaf and shoot material, however it is one of the largest ranges of values in the root data, and is overlapped with all but three of the other samples. The lowest concentration of phosphorus in root matter was recorded for the seedlings grown in 0A soils, followed by 40A and 40C soils. There was no phosphorus recorded in the 0A sample, although it may be a negligible concentration that was too small to be detected by the *Gallery Plus*.

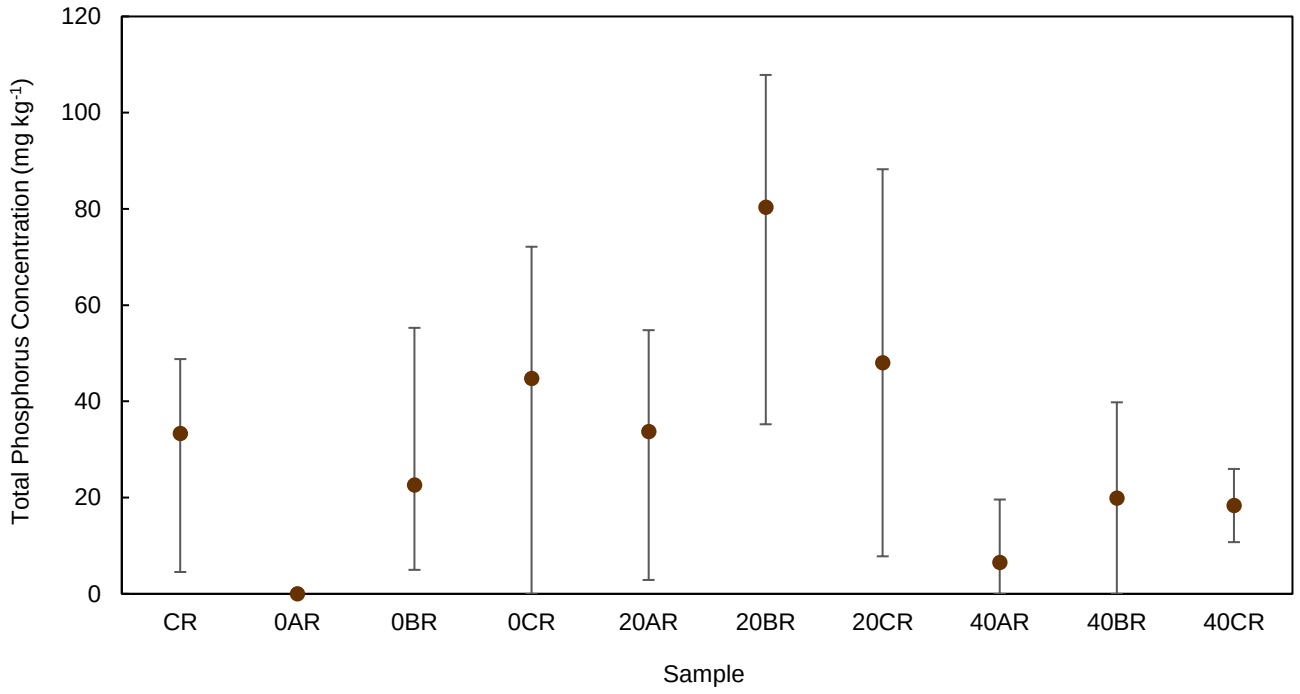


Figure 35 - Total phosphorus concentration in root matter of the samples, measured in mg kg^{-1}. The mean average total phosphorus concentration is indicated by the markers, with range bars used to illustrate the maximum and minimum values.

Although there are visible trends that can be observed in the data sets presented in Figure 33, Figure 34 and Figure 35 it is important that the data is analysed to test for statistical significance. Table 7 presents the results of the statistical testing for the total phosphorus concentrations of plant biomass, which is plotted in Figure 34. A t-test was used to test if the difference between the mean averages of two variables are statistically significant; a t-test can be run for equal or unequal variance in the data, and so an f-test was first conducted for each pair of variables, to establish the type of distribution in the data set. The f-test analysis is located in Appendix F and was used to choose the appropriate t-test for each of the pairs of variables analysed in Table 7. To identify statistical significance using a t-test, one or more of the conditions must be fulfilled. Of all the pairs of variables tested, none were found to have a statistically significant difference between them. Therefore, although some patterns are present within the dataset, it is not possible to draw firm conclusions from them given the lack of

statistical significance.

Table 7 - t-Test results for total phosphorus concentration in plant biomass. The t-Test seeks to identify if the mean values of the two variables/samples are significantly different. A two-tailed t-Test requires the results to satisfy at least one of the following criteria, in order to reject the null hypothesis: the t Stat < -t Critical two-tail or t Stat > t Critical two-tail. If either test is satisfied, then the null hypothesis ($\mu_1 - \mu_2 = 0$) is rejected, and it can be concluded that the variable means differ significantly.

Variable 1	Variable 2	t Stat	t Critical two-tail	t Stat < - t Critical two-tail	t Stat > t Critical two-tail
0AP	20AP	-1.20228	4.30265273	No	No
0AP	40AP	-1	4.30265273	No	No
20AP	40AP	0.023319	2.776445105	No	No
0BP	20BP	-0.80509	4.30265273	No	No
0BP	40BP	0.873067	4.30265273	No	No
20BP	40BP	1.004644	4.30265273	No	No
0CP	20CP	0.69263	2.776445105	No	No
0CP	40CP	1.500581	4.30265273	No	No
20CP	40CP	1.277525	4.30265273	No	No
0AR	20AR	-2.13902	4.30265273	No	No
0AR	40AR	-1	4.30265273	No	No
20AR	40AR	1.59268	2.776445105	No	No
0BR	20BR	-2.06164	2.776445105	No	No
0BR	40BR	-0.97467	4.30265273	No	No
20BR	40BR	-0.60349	4.30265273	No	No
0CR	20CR	-0.07812	3.182446305	No	No
0CR	40CR	0.889721	3.182446305	No	No
20CR	40CR	0.724708	4.30265273	No	No

7.0. DISCUSSION

The wide range of data generated in this research project can be collated to draw conclusions relating to the presence of phosphorus in soils from the karst critical zone of south west China, and how oxalic acid exudates influence phosphorus uptake by primary producers. These findings can be directly linked back to the hypotheses laid out in Section 4.0.

The results from the SEM EDS mapping can be used to draw firm conclusions about the nature of the Chenqi soils, and the presence and forms of phosphorus found within them. The Chenqi samples used in this research project were collected from soil pits in areas of abandoned farmland; prior to being abandoned, this land was cultivated, growing soybeans, maize and peanuts for both subsistence and commercial farming purposes. From personal communications between SPECTRA researchers and local farmers, it is understood that NPK fertilisers were used in abundance throughout this active agricultural period to increase soil solution phosphorus concentrations which would in turn improve crop yield. These were applied to the soil surface and either dug into the top layers of soil, or in the presence of crops left to infiltrate the soil when precipitation occurred. Data gathered as part of the SPECTRA project indicates that the use of fertilisers across southwest China has been increasing since the mid 1990s, and is set to continue its increase given the growing population across this region (Green et al., *in press*). Despite the increase in NPK fertiliser application across the south west China karst, the SEM EDS imaging and spectral analysis identified negligible concentrations of phosphorus in the A and C horizon samples. Although phosphorus-containing fertilisers were applied whilst this land was used for agriculture, legacy phosphorus does not appear to have been retained in the soils in non-bioavailable forms. The lack of phosphorus present from the SEM EDS imaging indicates that naturally-occurring phosphorus is limited in the Chenqi soils, in addition to there being no legacy phosphorus present from the application of mineral fertilisers in the past.

The Chenqi subcatchment and associated karst landscape are both highly weathered environments, which directly links to the phosphorus limitation identified from the SEM EDS imaging. Figure C1 in Appendix C shows an SEM EDS map of titanium in the A horizon of the Chenqi soils; there is an unusually high abundance of titanium within the soil sample, which indicates that the soil is highly weathered. Titanium, present in soil as titanium oxide, is highly resistant to weathering and is therefore considered a proxy indicator for whether a soil is highly weathered. As soils are weathered, concentrations of more soluble nutrients such as phosphorus decrease, whilst the proportion of titanium is seen to increase (Porder et al., 2007). When using titanium as a proxy for soil weathering, the whole soil profile must be analysed, to ensure that anthropogenic sources of titanium are not simply responsible for polluting the A horizon; unusually high concentrations of titanium at depth confirms that soils are weathered. A similarly high abundance of titanium is also present in the C horizon soils (see

Appendix D), which further proves the original hypothesis that soils in the Chenqi subcatchment are highly weathered, which is in-part responsible for the phosphorus limitation that has identified from this research. Phosphorus in soil is susceptible to leaching and erosion, resulting in depletion in highly weathered environments; karst regions are often characterised by their highly weathered soils, and have therefore been found to be limited with respect to phosphorus (Bull, 2005; Kertesz and Frossard, 2015).

Of the phosphorus that was identified using the SEM-EDS imaging, the majority was held in the form of iron-bound and aluminium-bound species. In the A-horizon, the dominant form of phosphorus was iron-bound, in addition to a small number of species that appeared to remain unbound. The iron-bound and aluminium-bound species identified in the A horizon have likely originated through the precipitation of $H_2PO_4^-$ and HPO_4^{2-} species with iron or aluminium cations (Pierzynski et al., 2005). Both iron and aluminium are found in natural abundance (82,000 ppm and 41,000 ppm respectively) in the Earth's crust and are subsequently found in high concentrations in many soils. SEM EDS analysis of calcium within the Chenqi soils showed the high concentrations of calcium species, which was expected given the origin of the soils being a karst environment underlain by limestone bedrock. Despite the high concentration of calcium species within the A horizon soils, Figure 24B highlighted the negligible concentrations of calcium-bound phosphorus species. In such karst environments, it would be expected that the majority of phosphorus species would be in the calcium-bound form, however in the A horizon soils sampled from Chenqi subcatchment iron-bound phosphorus was found to be in far higher concentrations than calcium-bound or aluminium-bound species. The unbound phosphorus species identified in the SEM-EDS analysis is likely held as either organic phosphorus or inorganic primary apatite minerals. As illustrated in Figure 1, organic phosphorus originates from detrital plant and animal matter, humic substances and microbial biomass (Cade-Menun et al., 2000; Condron et al., 2005), whilst inorganic primary phosphorus minerals take the form of apatitie originating from the weathering of the underlying bedrock (Bieleski, 1973; Dalai, 1977; Stevenson, 1986).

The results from the SEM EDS analysis of the A and C horizons of Chenqi soils are in accordance with the data generated from a sequential extraction previously conducted on the soil samples by a student completing a summer internship, Sheena Ramgulam (School of Earth Sciences, University of Bristol_, which is presented in Appendix B. The extraction used the method outlined by (Ruttenberg, 1992), and concluded that the most prevalent form of phosphorus in the Chenqi soils was iron-bound phosphorus This form is itself non-bioavailable, however there are known interactions between phosphorus phases that could have increased the concentration of bioavailable phosphorus in the soil solution. Given that the majority of phosphorus found in the Chenqi soils is iron or aluminium bound in nature, desorption can be assumed to be one of the key processes in maintaining bioavailable phosphorus in soil solution in Chenqi. Desorption sees the release of phosphorus from soil-bound metal ions, such as aluminium and iron, converting non-bioavailable species into organic bioavailable forms of phosphorus (Pierzynski

et al., 2005). This conclusion is drawn based on the understanding of phosphorus cycling that is laid out in the current literature, however there remains some interactions which are still poorly understood. For example, the factors which control desorption, adsorption and mineralisation of phosphorus have not yet been explored in full, making it difficult to draw firm conclusions on the species of phosphorus used most commonly by plants. These gaps in the overall phosphorus cycle make it difficult to understand how the interaction of different cycling processes alters depending on environmental pressures.

The data obtained from the soil fractionation also indicate very low concentrations of calcium-bound phosphorus species; this mirrors the results of the SEM EDS analysis, in that low densities and concentrations of calcium-bound phosphorus were identified in both the A and C horizons of the Chenqi soils. Despite it mirroring the SEM EDS analysis, the fractionation remains an interesting result given the high concentration of calcium within the soil sample, but negligible concentration of calcium-bound phosphorus species. There are a number of explanations for the findings from the SEM EDS and fractionation, in relation to the high iron-bound phosphorus concentration and low calcium-bound phosphorus concentration. Firstly, the findings may be explained by the nature of the environment in which they have been found. The Chenqi subcatchment in Guizhou is a highly-weathered karst environment, and it is possible that the state of weathering has influenced the phosphorus species found in the soil samples. Previous research indicates that in unweathered environments, inorganic phosphorus is more commonly associated with calcium and magnesium compounds; conversely, in highly weathered environments, iron-bound and aluminium-bound phosphorus compounds are the dominant fractions (Cross and Schlesinger, 2001; Ippolito et al., 2010; Lajtha and Bloomer, 1988; Lajtha and Schlesinger, 1988). Given the highly-weathered and degraded nature of the soils sampled from the Chenqi subcatchment, it is perhaps unsurprising that the majority of the soil inorganic phosphorus is held in iron bound compounds.

The findings from the fractionation and SEM EDS analysis may also be explained by the extraction method used to generate the fractionation data. Although the Ruttenberg (1992) fractionation method is widely used for phosphorus analysis in soils and sediments, it has been critiqued for generating phosphorus concentrations up to 14% lower than the Hedley fractionation scheme (1982) (Levy and Schlesinger, 1999; Ruttenberg, 1992). Although this bias may also increase the concentration of iron-bound phosphorus in the soil sample, it may also work to increase the calcium-bound and aluminium-bound phosphorus species. There are a large range of phosphorus extraction and fractionation method which can be employed for different soil types (e.g. Chang and Jackson, 1957; Condron et al., 1990; Hedley et al., 1982; Hieltjes and Lijklema, 1980; Williams et al., 1967). Extensive research has been conducted into the limitations of these different extraction methods; Williams et al (1980) tested a wide range of extraction methods and found that some phosphorus-containing compounds are insoluble with all reagents used in the extraction methods. For example Crandallite, a calcium- and aluminium-bound

phosphorus compound) remains insoluble in all reagents used. Ruttenberg also uses these tested reagents within the phosphorus extraction method and therefore it must be considered that some of the calcium-bound and aluminium-bound phosphorus was held in compounds such as Crandallite, which are insoluble with respect to the extraction reagents. This may provide an explanation for lower concentrations of calcium-bound and aluminium-bound phosphorus than would perhaps be expected for a soil sampled from a karst environment.

Further to the SEM EDS analysis and the fractionation data, the concentration of phosphorus in plant biomass can be used as a reflection on the availability of phosphorus in soils and provide a stronger understanding of nutrient availability in complex, highly weathered karst environments. The data produced through total phosphorus analysis using the *Gallery Plus* autoanalyser was tested for statistical significance using t-tests, which confirm whether the difference between the mean averages of two variables is statistically significant or not. None of the combinations of soil treatments tested for variability registered any statistical significance, and therefore all interpretation and analysis of the raw data can only be conducted via identifying visual trends, with a hope to researching these ideas further in future work to generate statistically significant datasets.

A general trend observed from the dataset presented in Figure 30 is the relationship between the phosphorus concentration of leaves and shoots compared to the total phosphorus concentration found in plant roots. In the case of all but one soil treatment, the root matter had a consistently higher concentration of total phosphorus than the corresponding leaves and shoots. Such a pattern could be attributed to several factors, in particular the accumulation of phosphorus in roots, when plants are grown in phosphorus-limited soils. Chapin (1980) noted that in plants grown in soils considered to be nutrient deficient, phosphorus concentrations were found to be higher in the roots than the above-ground biomass. It is thought that this is an adaptation by some plants, to encourage the growth of larger root systems, to explore more of the soil profile to acquire much-needed phosphorus (Chapin, 1980). This is backed up by the results observed in the plant health and growth quality qualitative assessment, the results of which are presented in Section 6.2. Phosphorus deficiency in plants is usually highlighted by a purple-brown mottling on the leaves and stems of the plant, in addition to the stunting of growth in more serious cases of phosphorus limitation. All seedlings grown on Chenqi soils, including those treated with oxalic acid, showed obvious signs of phosphorus limitation. Plants had purple and brown mottling across the leaf surface, in addition to a dark purple discolouration occurring at the base of the stems and on new shoots. In contrast, the control seedlings, grown on autoclaved potting compost known to contain an adequate phosphorus concentration, presented no symptoms of phosphorus limitation, as can be seen in Figure 25. These findings link to several of the hypotheses originally laid out in Section 4.0.; it can be accepted that the Chenqi soils are limited with respect to phosphorus, and that this limitation has likely caused for the concentrations in plant roots to be greater than the

concentrations of phosphorus found in leaves and shoots. This perhaps provides an insight into the adaptive capacity of plants growing in such nutrient-poor regions, and how simple methods of qualitative analysis can be used in partnership with biogeochemical techniques to answer complex research questions.

A further interesting observation from both the analysis of plant health and of total phosphorus concentration, is that soils treated with oxalic acid still cultivated seedlings that presented signs of phosphorus limitation and did not contain significantly higher concentrations of phosphorus than those soils which remained untreated. The purple-brown mottling indicative of phosphorus limitation was present on seedlings grown in soils treated with 20mM and 40mM oxalic acid, despite the known links between oxalic acid exudates and increased uptake of phosphorus by primary producers (Panhwar et al., 2013). The statistical analysis of the data generated from total phosphorus analysis shows there not to be a statistically significant difference between the phosphorus concentration of plants grown in untreated soils and those treated with either 20mM or 40mM oxalic acid. In the research conducted by Panhwar et al (2013), treatments of 20mM oxalic acid was found to be most effective in increasing the uptake of phosphorus by plants, however the soils used were not limited with respect to phosphorus. The Chenqi soils are known, because of this research, to be limited with respect to phosphorus; it is therefore important to consider that perhaps the Chenqi soils are simply too severely phosphorus limited for treatments of oxalic acid to significantly increase phosphorus uptake by primary producers. Given the very low abundance of phosphorus found using the SEM EDS imaging, and the low concentrations found by sequential extraction work (conducted by Sheena Ramgulam, School of Earth Sciences, University of Bristol), it is possible that phosphorus concentrations in the Chenqi soils are too depleted to be positively affected by oxalic acid treatments. If so, then other methods of management for such nutrient-limited soils will require developing in the future, as applications of oxalic acid may not mobilise sufficient phosphorus for high-quality crop cultivation.

A further explanation for statistical analysis results, is despite the findings in the current literature, oxalic acid may not be the optimum organic acid for the Chenqi soils. Although oxalic acid is identified as the most effective organic acid in Panhwar et al (2013), it should be recognised that other organic acids could produce more bioavailable phosphorus in severely nutrient-limited soils. Previous research has sought to identify the most effective chelating compound to be added to soils for increasing phosphorus uptake (Hue, 1991; Panhwar et al., 2013), however no studies have focused upon karstic soils. The specific chemical composition of karstic soils, and severely limited concentration of phosphorus found in Chenqi may result in oxalic acid not being the most effective organic acid or chelating compound for soils in the karst region of south west China. Furthermore, the concentrations of oxalic acid used for soil treatments may have been too low to chelate phosphorus compounds, which resulted in the physical indications of phosphorus limitation observed in the *Erigeron acris* grown in the suite of experiments.

The concentrations of oxalic acid chosen for dosing Chenqi soils were based on research conducted by Panhwar et al (2013), which explored the addition of LMWOAs to rock phosphate to increase phosphorus uptake by aerobic rice species. Although Panhwar et al concluded that 20mM oxalic acid was the most effective LMWOA for increasing total phosphorus uptake by plants, it is important to consider how this research can be transferred to this project. Chenqi subcatchment is part of the wider south west China karst, and is characterised by calcium-carbonate rich soils; these soils have a high buffering capacity, and therefore may require a higher concentration of LMWOAs to be applied in order to mobilise phosphorus fractions for uptake by biota. In contrast, the rock phosphate used by Panhwar et al was less calcium-rich, and therefore is likely to have a lower buffering capacity for LMWOAs; this would result in lower concentrations of LMWOAs acting to mobilise more phosphorus than would be mobilised in soils with a high buffering capacity. It should therefore be considered that increased concentrations of oxalic acid used in dosing of the Chenqi soils could result in an increased mobilisation of phosphorus compounds and would therefore produce plants with a higher total biomass than was observed in plants grown in 20mM or 40mM oxalic acid. It is also likely these plants would present with fewer or no symptoms of phosphorus limitation, such as the purple-brown mottling on the leaves which was observed in the plants grown in this suite of experiments.

One of the key research aims of this research and of the overarching SPECTRA project is to better understand ecosystem resilience and restoration, and to develop methods by which karst critical zones, such as Chenqi subcatchment, can be managed for socio-economic benefit. Therefore, analysis of the change in total plant biomass over the duration of the growing period is important, as it links back to the primary land-use across Chenqi, that of subsistence and commercial arable farming. The results presented in Figure 32 indicate there is no obvious relationship between the concentration of oxalic acid applied in soil treatments and the change in total plant biomass. The control sample, C, saw no change in biomass over the cultivation period, whilst all but two of the Chenqi soils saw an increase in plant biomass for their associated *Erigeron acris* seedlings. The negligible change in average biomass for the control seedlings is somewhat unexpected, given that the potting compost used as a control was not limited with respect to phosphorus. The negligible change in biomass may be explained by the methodology; when potting up seedlings between the seed compost and the final soil treatments, excess soil was removed by hand, to prevent possible root damage that may occur through washing. Traces of soil in the roots could have increased the ‘before’ mass, which would not have been captured in ‘after’ mass measurements, as seedlings were washed to remove all soil. Therefore, the control seedling biomass may have increased but gone unrecorded, due to the inclusion of minor soil traces in the ‘before’ plant mass values. Statistical analysis of the data used to assess if increasing oxalic acid concentrations could increase plant biomass, as a result of increasing bioavailable phosphorus for uptake by primary producers. The t-test analysis used shows there to be no statistically significant

difference between the mean biomass changes of any of any two treatments, however this may be linked to the large range in values generated for each of the soil treatments, resulting in overlapping range bars for many of the soil treatments. A larger number of repeats could help to isolate outliers, producing smaller data ranges, which may remove the noise from the data and highlight statistically significant results.

8.0. LIMITATIONS AND FUTURE WORK

The research conducted in this project is subject to several limitations, which have subsequently generated ideas for novel future research. The primary limitation of this research is that plant growth experiments were only conducted using autoclaved soil samples, as a result of the DEFRA licence assigned to the soils imported to the UK from Chenqi catchment. The licence outlines that the soils cannot be used for plant cultivation, unless they have been autoclaved or otherwise sterilised to produce unlicensed material. Non-autoclaved Chenqi soils could only have been used for plant cultivation experiments if conducted within a fully-licensed facility; such a facility was unavailable at the University of Bristol. Conducting the same experimental procedure using non-autoclaved soils would provide an interesting comparison to the data generated from this research. For example, if differences in total phosphorus concentration of seedlings grown in soils with no oxalic acid (0A, 0B and 0C samples) were found between autoclaved and non-autoclaved soils, it could highlight the action of mycorrhizal fungi in non-autoclaved soils. The University of Exeter is currently in the process of obtaining a licence from DEFRA to conduct this experimental sequence using non-autoclaved soils, starting in Autumn 2018; the results of the experiments using autoclaved and non-autoclaved soils will both feed back into the SPECTRA project, where they will be used to inform on potential management strategies to increase phosphorus availability in karst soils.

In future, an additional suite of experiments should be run using the Chenqi soils and increased concentrations of oxalic acid. The calcium-carbonate rich nature of the soils sampled from Chenqi results in a high buffering capacity, which may act to neutralise the oxalic acid treatment applied to soils. This would prevent the chelation of phosphorus species, and therefore could have influenced the results observed. In future, higher concentrations of oxalic acid should also be used to account for the buffering action of the soils. Such an experiment could include concentrations ranging from 0mM to 100mM, in either 10mM or 20mM increments. Depending on the volume of soil available for planting experiments, the LMWOA used could also be compared; in Panhwar et al (2013) a range of organic acids were compared, however this experiment did not focus on karst soils and therefore there is the potential for a comparison of LMWOA application in nutrient-limited karstic soils.

Further to the aforementioned suite of experiments using non-autoclaved soils, research could be conducted into identifying the species of phosphorus being acquired by primary producers in both autoclaved and non-autoclaved soils. This comparison assumes that the non-autoclaved soils maintain a presence of mycorrhizal fungi, which may exude organic acids to break down non-bioavailable phosphorus forms. A sequential extraction of phosphorus, according to the Ruttenberg (1992) method, could be conducted both before and after the cultivation of *Erigeron acris* seedlings, to determine the

change in concentrations of different forms of phosphorus. This may identify both the species of bioavailable phosphorus most readily acquired by plants, in addition to the non-bioavailable forms most readily converted to bioavailable species.

A further limitation of the research was only analysing one sample from the A horizon and the C horizon of the Chenqi soils. The clay-rich nature of the Chenqi soils resulted in difficulty achieving complete impregnation by the epoxy resin. The method for impregnation was altered during the research project, but more work could be done in future on impregnating clay-rich soils, to allow for analysis of more samples of the Chenqi soils. This would ensure that the results were of a representative sample of the soil, rather than highlighting localised chemical or geological phenomenon.

Furthermore, this analysis only examined soils samples collected from abandoned farmland in Chenqi; these soils were used throughout this research but could be made more comprehensive through analysing samples from the other land-use types: primary forest, secondary forest and cultivated farmland. Through comparing SEM EDS data for each land-use, understanding of phosphorus cycling interactions in varying land-use types could be improved, which could result in more effective management techniques for karst regions such as Chenqi.

A limitation that must be considered when drawing conclusions about phosphorus cycling in karst regions such as Chenqi, is the highly-specific nature of the research conducted, and therefore its applicability to other regions must be critically evaluated. Through only analysing soils from abandoned farmland in Chenqi, it is difficult to draw conclusions about the subcatchment as a whole, given that land-use type is known to influence phosphorus cycling and availability. By including soil samples from the primary and secondary forest, in addition to active farmland, a more comprehensive understanding of phosphorus cycling in karst may be established. It is hoped that research being conducted by other SPECTRA project researchers in the UK and China will be fed back into the project as a whole and will be used to better determine phosphorus cycling and interactions across the karst critical zone of south west China.

9.0. CONCLUSION

A number of conclusions can be drawn based on the research conducted in this project, which can be linked back to the research aims and objectives and hypotheses laid out in Section 3.0 and 4.0 respectively. These findings can be used to answer important questions about the karst critical zone of south west China, in addition to being fed back into the SPECTRA project and used to develop methods of management for such nutrient-poor regions.

A range of quantitative and qualitative research methods were developed to answer the primary research question: “Is phosphorus uptake by primary producers in calcareous soils from karst regions controlled by organic acid exudates from mycorrhizal fungi?”. Plant-based experiments were conducted, with the plant health monitored, in addition to changes in biomass recorded over the growth period. Total phosphorus concentrations of the plant matter were analysed, to try to prove a number of hypotheses relating to phosphorus availability and the action of oxalic acid exudates in karstic soils. These research experiments all used soils collected from Chenqi subcatchment in Guizhou Province, south west China, from an area of abandoned farmland that was once used for subsistence and commercial farming.

The results of this research suggest that oxalic acid exudates from mycorrhizal fungi do not result in a significant increase in phosphorus uptake by primary producers. Results suggest that oxalic acid does not cause a significant increase in plant biomass when compared to soils with no oxalic acid treatment applied. There is also no significant difference in the total phosphorus concentrations in plants grown in soils treated with oxalic acid, compared to those which remain untreated. Furthermore, there is evidence to suggest that the soils in Chenqi are severely limited with respect to phosphorus, and that this nutrient deficiency impacts upon the growth quality and overall health of plants cultivated in Chenqi. SEM EDS analysis detected low concentrations of phosphorus in all soils horizons, and observations of plant health during the plant growth experiments indicate that plants grown in Chenqi soils are deficient in phosphorus. These results, coupled with the understanding that oxalic acid does not significantly increase phosphorus uptake, perhaps suggest that the soils in Chenqi subcatchment are too severely limited with respect to phosphorus to be affected by the addition of oxalic acid. Although low concentrations were identified using SEM EDS analysis, it is likely to be insufficient for maintaining healthy plant growth.

This research project was limited by several factors, most importantly the licencing attached to the Chenqi soils, and subsequently the requirement that they must be autoclaved prior to any experimental work. This limited the exploration into the specific role of mycorrhizal fungi and resulted in the addition of oxalic acid as a proxy for the natural organic acid exudates released from mycorrhizal fungi to increase phosphorus bioavailability. Further research is planned at a licenced facility at the University of Exeter, which will use the same experimental design as this project, but will use non-autoclaved soils,

in an effort to identify the presence and role of naturally-occurring mycorrhizal fungi in Chenqi soils. This research, in addition to the continued works at the University of Exeter, will feed back into the SPECTRA project findings.

The research laid out in this thesis is part of the overarching SPECTRA project, which plans to use biogeochemical research techniques to improve the resilience and recovery of nutrient-limited karst landscapes across south west China. Nutrient-limited karst environments, such as those in and around Chenqi catchment, are under continued environmental, social and economic strain. It is only through projects such as SPECTRA that insight into the complex interactions of nutrients within desertified karst regions can be improved. The future of karst critical zones relies upon increased research into how they can be managed, to provide a livelihood for those currently living there, whilst not depleting or degrading the natural resources so that future generations are impacted negatively.

10.0. REFERENCES

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11.0. APPENDICIES

APPENDIX A: SAMPLE CODES FOR TOTAL PHOSPHORUS CONCENTRATION ANALYSIS

Table A1 – Sample codes used for identifying individual plant samples for total phosphorus analysis using the Gallery Plus automated photometric analyser

Code	Soil Horizon	Oxalic Acid Conc (mM)	Repeat Number	Plant/Root?
C1P	Potting Compost	0	1	Plant
C1R				Root
C2P			2	Plant
C2R				Root
C3P			3	Plant
C3R				Root
0A1P	A		1	Plant
0A1R				Root
0A2P			2	Plant
0A2R				Root
0A3P			3	Plant
0A3R				Root
0B1P	B		1	Plant
0B1R				Root
0B2P			2	Plant
0B2R				Root
0B3P			3	Plant
0B3R				Root
0C1P	C		1	Plant
0C1R				Root
0C2P			2	Plant
0C2R				Root
0C3P			3	Plant
0C3R				Root
20A1P	A	20	1	Plant
20A1R				Root
20A2P			2	Plant
20A2R				Root
20A3P			3	Plant
20A3R				Root
20B1P	B		1	Plant
20B1R				Root
20B2P			2	Plant
20B2R				Root
20B3P			3	Plant

20B3R				Root
20C1P	C		1	Plant
20C1R				Root
20C2P			2	Plant
20C2R				Root
20C3P			3	Plant
40A1P	A	40	1	Plant
40A1R				Root
40A2P			2	Plant
40A2R				Root
40A3P			3	Plant
40A3R				Root
40B1P	B		1	Plant
40B1R				Root
40B2P			2	Plant
40B2R				Root
40B3P			3	Plant
40B3R				Root
40C1P	C		1	Plant
40C2P				Root
40C2R			2	Root
40C3P			3	Plant
40C3R				Root

APPENDIX B: SEQUENTIAL EXTRACTION OF PHOSPHORUS

The following data is the result of a sequential extraction conducted by Sheena Ramgulam, a summer intern student in the School of Earth Sciences, University of Bristol. The sequential extraction was conducted using the method outlined in Ruttenberg (1992).

Table B1 – Raw data from sequential extraction of phosphorus, conducted by Sheena Ramgulam, School of Earth Sciences, University of Bristol, according to the method proposed by Ruttenberg (1992). All values are given in mg L^{-1}.

	Extraction 1	Extraction 2	Extraction 3	Extraction 4	Extraction 5
	Exchangeable or loosely-sorbed phosphorus	Iron-bound phosphorus	Authigenic apatite, calcium-bound phosphorus and biogenic apatite	Detrital apatite and other inorganic phosphorus	Organic phosphorus
	Concentration (mg L^{-1})				
A Horizon	0.001471769	153.3291667	0.006815151	0.036296757	0.002870036
B Horizon	0.008589377	180.0097222	0.003137236	0.008053388	0.003537185
C Horizon	0.004431373	167.9	0.002235329	0.007928853	0.003463991

Table B2 – Sequential extraction of phosphorus, with data displayed as a percentage of the total phosphorus.

	Extraction 1	Extraction 2	Extraction 3	Extraction 4	Extraction 5
	Exchangeable or loosely-sorbed phosphorus	Iron-bound phosphorus	Authigenic apatite, calcium-bound phosphorus and biogenic apatite	Detrital apatite and other inorganic phosphorus	Organic phosphorus
	Proportion of Total Phosphorus (%)				
A Horizon	0.000959578	99.96906066	0.004443409	0.023665117	0.001871235
B Horizon	0.004771	99.98704839	0.001742589	0.004473283	0.001964742
C Horizon	0.002639009	99.98924502	0.001331202	0.004721859	0.002062906

APPENDIX C: SEM EDS MAPS – A HORIZON

SEM EDS elemental mapping of sample of A horizon soil collected from Chenqi in 2016. The following elemental analysis was not directly related to the presence of phosphorus within the soils, but instead provides additional information about the nature of the karst soils.

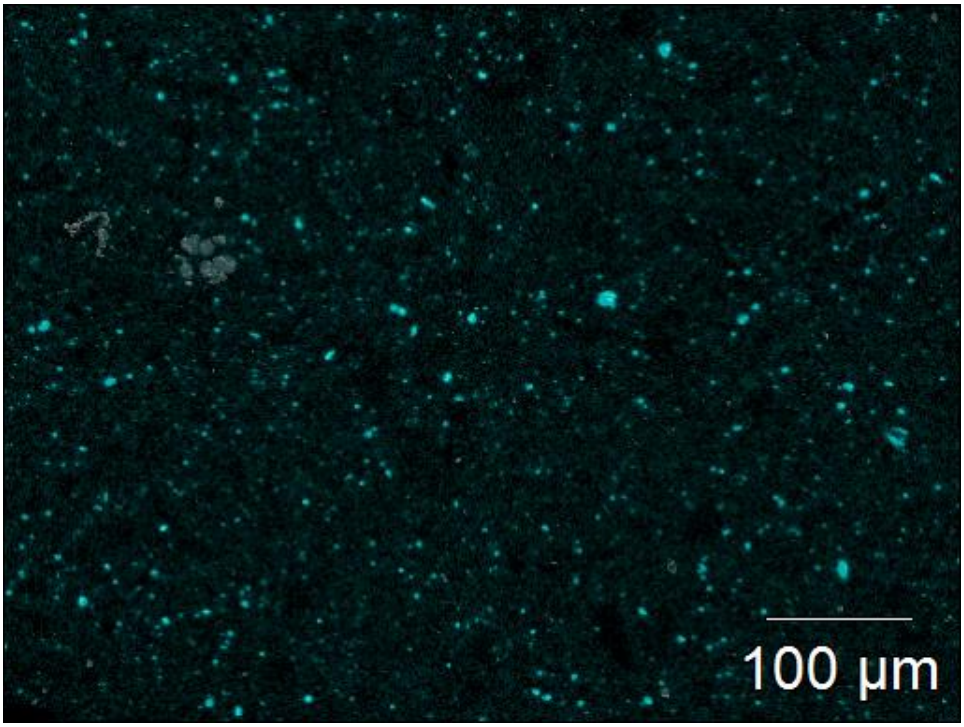

Figure C1 – SEM EDS map of titanium present within the A horizon of the soils collected from Chenqi in 2016. Light blue areas are those with a high titanium concentration.

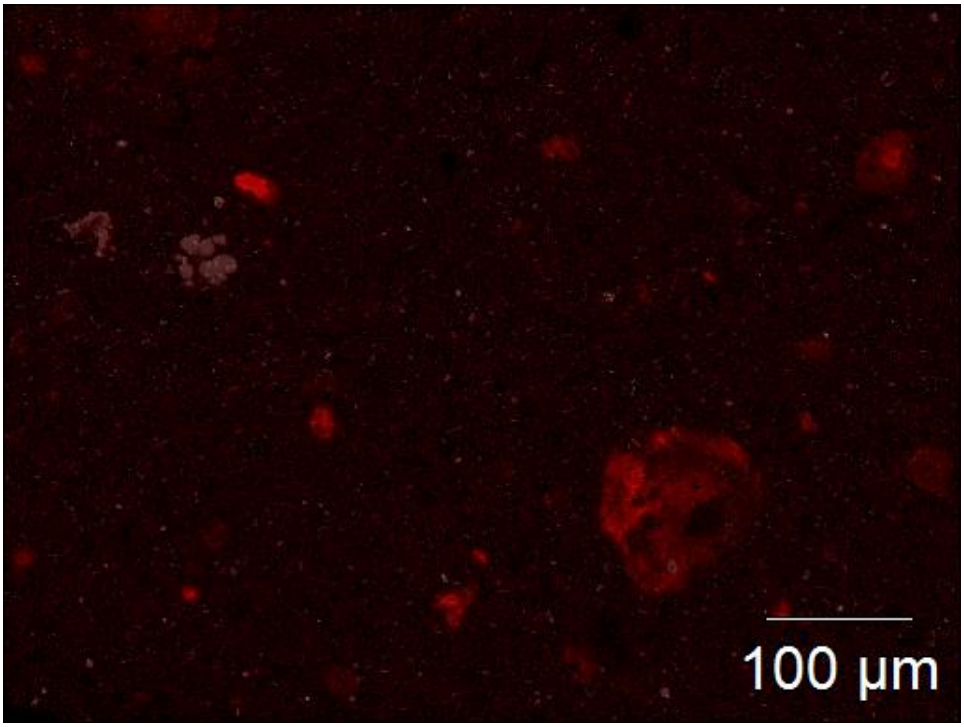

Figure C2 – SEM EDS maps of manganese in the A horizon soil collected from Chenqi subcatchment in 2016. Areas of high manganese concentration are a lighter red/pink colour, while dark red/brown colours indicate background or negligible concentrations of manganese.

APPENDIX D: SEM EDS MAPS – C HORIZON

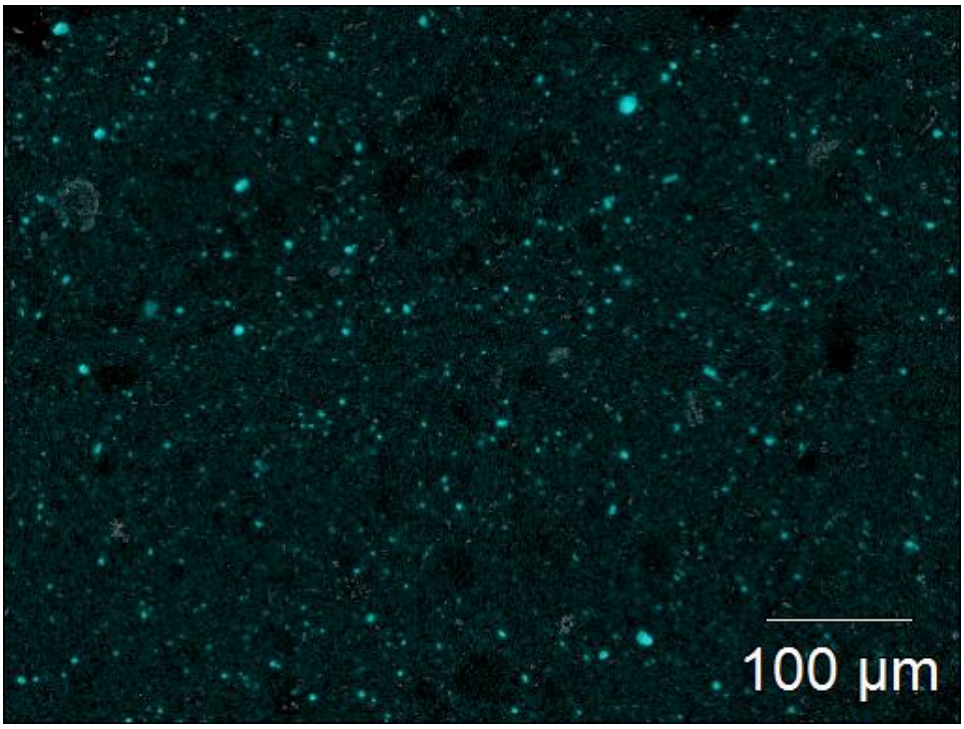

Figure D1 – SEM EDS analysis of titanium in the C horizon of the Chenqi soils. Areas of bright blue represent high concentrations of titanium, whilst the darker blue/grey colour indicates background concentrations of titanium.

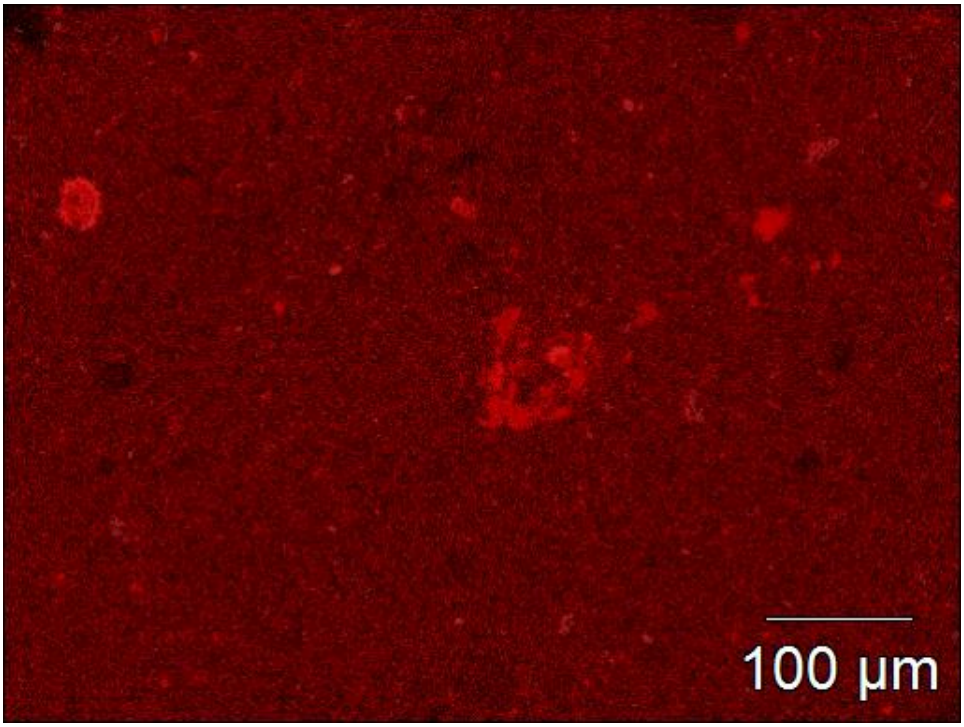

Figure D2 – SEM EDS analysis of manganese in the C horizon of the Chenqi soils. Areas of light red or pink indicate high concentrations of manganese, whilst the darker red show background levels of manganese in the sample.

APPENDIX E: F-TEST RESULTS – PLANT BIOMASS

Table E1 – F-test results for total plant biomass change. F-tests were completed to establish if the variance between two samples was equal or unequal, and therefore the type of two-tailed t-test required. If F > F-Critical, then the null hypothesis ($\sigma 1^2 = \sigma 2^2$) is rejected, and the variances of the two samples considered unequal.

Variable 1	Variable 2	F	F-Critical	Results
0A	20A	21.92228	19	Unequal
0A	40A	2.279568	19	Equal
20A	40A	9.616856	19	Equal
0B	20B	14.67548	19	Equal
0B	40B	1.650098	19	Equal
20B	40B	8.893699	19	Equal
0C	20C	1.299001	19	Equal
0C	40C	7.489277	19	Equal
20C	40C	9.728578	19	Equal

APPENDIX F: F-TEST RESULTS – TOTAL PHOSPHORUS CONCENTRATION

Table F1: F-test results for total phosphorus concentration data. F-tests were completed to establish if the variance between two samples was equal or unequal, and therefore the type of two-tailed t-test that would be required. If F > F-Critical, then the null hypothesis ($\sigma 1^2 = \sigma 2^2$) is rejected, and the variances of the two samples can be considered unequal.

Variable 1	Variable 2	F	F-Critical	Result
0AP	20AP	65535	19	Unequal
0AP	40AP	65535	19	Unequal
20AP	40AP	1.286876	19	Equal
0BP	20BP	11.5668	19	Equal
0BP	40BP	33.68275	19	Unequal
20BP	40BP	389.6016	19	Unequal
0CP	20CP	2.19132	19	Equal
0CP	40CP	130.5089	19	Unequal
20CP	40CP	59.55722	19	Unequal
0AR	20AR	65535	19	Unequal
0AR	40AR	65535	19	Unequal
20AR	40AR	5.425126	19	Equal
0BR	20BR	3.743963	19	Equal
0BR	40BR	1.542669	18.51282	Equal
20BR	40BR	2.426939	199.5	Equal
0CR	20CR	3.462452	18.51282	Equal
0CR	40CR	585.6336	199.5	Unequal
20CR	40CR	2027.728	161.4476	Unequal